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Expression in Breast Cancer Metastasis

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13. ABSTRACT (Maximum 200 Words) In the normal breast, hepatocyte growth factor (HGF), also known as scatter factor, is expressed primarily by stromal cells, while epithelial cells express the HGF receptor, Met. Thus, epithelial cells exhibit a tight paracrine loop regulating HGF-dependent responsiveness. In invasive human breast carcinomas, HGF and Met are frequently over-expressed, thereby establishing an autocrine HGF/Met signaling pathway and promoting tumour cell invasion. However, the mechanisms leading to aberrant expression of HGF in carcinoma cells are not known. We previously demonstrated a co-operative effect of c-Src tyrosine kinase and Stat3 in the activation of HGF transcription in mammary carcinoma cells. In the present report, we have shown that non-malignant breast epithelial cells over-expressing activated c-Src and Stat3 exhibit increased activation of Stat3, HGF transcription, and cell scattering. Mutational analysis of the HGF promoter revealed a novel Stat3 binding site at nt -95, which is required for the c-Src/Stat3 co-operative effect. Our results delineate a novel c-Src/Stat3-dependent mechanism that regulates HGF transcription in breast carcinomas. This study could lead to novel strategies inhibition of HGF gene expression in tumour cells with minimal effects on normal HGF/Met function.				
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PREFACE:

This three year training award was initially held by Dr. Wesley Hung who was a postdoctoral fellow in Dr. Elliott's laboratory from 1998-2000. The final year of this award (2002-2003) was held by Dr. Joanna Wojcik, who was an MSc graduate student in Dr. Elliott's laboratory. Dr. Christopher Mueller was a co-supervisor of this project.

INTRODUCTION:

Breast cancer is the most common malignancy among women in Canada (1). However, there are no established prognosticators of metastatic disease among node-negative breast cancer patients. Recently, Rimm *et al.* (2) have shown that high expression of Met (cytoplasmic domain) in 324 node-negative breast cancer patients is associated with poor clinical outcomes and is distinct from Erb-B2 positive tumors. In addition, we (3,4) have previously shown strong co-expression of Met and its ligand, hepatocyte growth factor (HGF), in the migrating tumor front of invasive human breast cancers. Moreover, autocrine HGF/Met signaling is causally linked to tumorigenesis (5,6), and could therefore be an effective target for anti-cancer therapy, similar to targeting of Erb-B2 with herceptin (7).

Since HGF is not expressed in normal breast epithelial tissues, we have examined signalling mechanisms involved in upregulation of HGF expression in breast carcinomas, considering the possibility that this event may be relevant for prognostication and treatment strategies. In the present study, we have shown that Signal Transducer and Activator of Transcription 3 (Stat3) acts co-operatively with c-Src to activate transcription of *HGF* in mammary epithelial cells (i.e. upstream of HGF/Met), thereby contributing to autocrine activation of Met, and enhanced cell scattering (App. I & II). Since c-Src and Stat3 are over-expressed and activated in most human breast cancers (8,9), c-Src/Stat3 activation may be a key step in the over-expression of HGF and acquired autocrine HGF/Met signalling in epithelial cells, thereby contributing to mammary tumorigenesis.

BODY:

Original Hypothesis and objectives:

We hypothesize that establishment of an autocrine HGF loop and sustained activation of Met in breast carcinoma cells is pivotal in the transition from non-malignant to malignant epithelial growth. Based on this premise, increased *HGF* transcription and post-translational activation of HGF may be an important indicator of breast cancer progression. The transcription factors that control HGF expression and the signalling molecules that regulate the activity of these transcription factors may provide useful targets for new therapeutic treatment of breast cancer.

Therefore two **objectives** were proposed:

- 1) To examine transcriptional and post-transcriptional regulation of HGF expression in non-malignant and malignant breast epithelial cells;
- 2) To determine the role of Ras, PI3-kinase and c-Src (known to affect HGF-dependent functions) in HGF expression in breast carcinoma cells.

Objective 1)

As a first approach, we examined HGF mRNA and protein expression in a mouse epithelial cell line HC11 and a mammary carcinoma cell line SP1. Using a semi-quantitative RT-PCR assay to detect HGF mRNA, we showed significant expression of HGF mRNA in SP1 carcinoma cells compared to HC11 cells. Likewise high levels of HGF protein are secreted by SP1 carcinoma cells but not by HC11 epithelial cells (App. I & II). We further demonstrated that an inhibitor of c-Src family kinases (PP2) or expression of a dominant negative mutant of c-Src (SRC-RF) reduced the level of HGF mRNA and protein in SP1 carcinoma cells. Conversely, expression of an activated mutant of c-Src enhanced HGF mRNA and protein expression. Based on these findings, we focused the project on elucidating the mechanism by which c-Src regulates HGF expression in carcinomas cells. This work was carried out by Dr. Wesley Hung.

Objective 2)

To study transcriptional regulation of *HGF* we constructed a series of deletion mutants of the *HGF* promoter and inserted these mutants into luciferase reporter constructs. Using a transient co-transfection system, we demonstrated that *HGF* transcription (measured by luciferase activity) was modulated by c-Src activity. Furthermore, we identified a c-Src responsive region of the promoter at nt -254 to -70 in the *HGF* promoter. These results indicate that a c-Src-dependent pathway regulates HGF expression by modulating *HGF* transcription activity.

Analysis of the *HGF* promoter sequence revealed three putative Stat3 consensus sites in the c-Src responsive region (Fig. 1). Since Stat3 has been shown to act downstream of c-Src in the regulation of gene transcription (10), we examined whether Stat3 was involved in c-Src-dependent *HGF* transcription activity. We demonstrated a co-operative effect of c-Src and Stat3 in the activation of *HGF* transcription in SP1 breast carcinomas as well as HC11 epithelial cells (Fig. 2). Changes in c-Src kinase activation were shown to affect Stat3 activity through its phosphorylation of Stat3-Tyr705 and DNA binding to specific Stat3 consensus sites on the *HGF* promoter. This work was carried out by Dr. Wesley Hung and Joanna Wojcik (App. I & II).

In the next stage of the project, Dr. Joanna Wojcik examined the mechanism of c-Src-dependent Stat3 regulation of *HGF* transcription and of the transformed phenotype of mammary epithelial cells. Stable co-expression of activated c-Src and Stat3 caused increased *HGF* transcription, concomitant with marked cell scattering in breast epithelial cells (App. III). A putative Stat3 site at nt -95 was identified, as defined by the palindromic structure 5'-TTCCC^G/_G^T/GAA-3', which was shown to selectively bind to protein complexes containing Stat3 (11,12). Using a mutational approach we demonstrated that a consensus site at nt -95 (relative to the transcription start site) is responsible for the observed co-operative effect of c-Src and Stat3 in regulating *HGF* transcription (Fig. 3). DNA-protein binding studies further demonstrated that this site has high affinity for a Stat3-containing complex (Fig. 4).

The mechanism of c-Src/Stat3 regulation of *HGF* transcription through the nt -95 consensus site is distinct from previously identified mechanisms of *HGF* transcription

regulation through the Il-6 binding protein response element (at nt -211) (13) or the estrogen receptor response element (at nt -872) (14). Thus the signalling molecules c-Src/Stat3 appear to regulate *HGF* promoter activity preferentially in carcinoma cells. This signalling pathway could potentially define a new level of tumour specificity that might be associated with aberrant HGF expression in breast cancer. A manuscript describing this work has been submitted (App. III).

KEY ACCOMPLISHMENTS:

- Development of assays for detection of *HGF* transcription and mRNA and protein expression in mammary epithelial and carcinoma cells.
- First demonstration of a c-Src/Stat3 pathway that stimulates expression of HGF mRNA and protein breast in epithelial cells and carcinomas. This process may be a key step in early stage breast cancer.
- Identification of a novel Stat3 consensus site (at nt -95) in the *HGF* promoter that regulates c-Src/Stat3 transcription in mammary carcinoma cells.
- Proposition of a model for c-Src/Stat3-dependent regulation of HGF expression in invasive breast cancer. This pathway is a potential target for anti-cancer therapy in breast cancer metastasis.

REPORTABLE OUTCOMES:

- Published one research paper and one review article on this work. One manuscript submitted for publication (see App. I-III).
- Presented three abstracts of this work (see App. IV-V).
- Applied for funding from the Canadian Breast Cancer Research Initiative and the Canadian Institutes of Health Research for continuation of this work (PI: Bruce Elliott, PhD; CI: Christopher Mueller, PhD.). "Targeting a novel activating function of Stat3 in autocrine HGF expression in invasive breast cancer" These grants are pending.
- The PI (Joanna Wojcik) successfully completed an MSc defense, and received an MSc degree in Pathology in the summer of 2003. The Thesis was entitled "A novel activating function of Stat3 on the *HGF* promoter in mammary carcinoma cells".

CONCLUSIONS:

Two main objectives of this project were addressed in this study: 1) to assess HGF mRNA and protein levels in mammary epithelial and carcinoma cells and 2) to elucidate signal transduction pathways involved in the regulation of HGF expression during mammary tumor progression.

Whereas HGF expression is under tight negative regulation in breast epithelial cells (15), increased HGF expression frequently occurs in breast carcinomas, and is often associated with loss of epithelial differentiation and polarity, tumour invasiveness and metastatic behaviour (3,16,17,18). This capacity of carcinoma cells to express both HGF and its receptor results in activation of an autocrine HGF loop, but the molecular mechanisms responsible for increased HGF expression in malignant cells remain largely unknown. In the present study, we show that c-Src and Stat3 can act cooperatively to induce *HGF* transcription in mammary epithelial cells, and that the c-Src/Stat3 responsive region plays a critical role during this activation. We have further identified a novel Stat3 binding site at nt -95 of the *HGF* promoter. These observations suggest that the lack of c-Src kinase activity and the low level of activated Stat3 may be responsible for limiting *HGF* transcription in epithelial cells of the breast. Thus, overriding the tight repression of *HGF* transcription would require at least two events: increased activation of c-Src and over-expression of Stat3, with the latter being phosphorylated at Tyr-705 and activated by the former (19).

The mechanisms that lead to over-expression and activation of Stat3 and c-Src in tumour cells are not clearly known, but most likely involve multiple oncogenic changes. Both molecules are critical downstream effectors of Met and are required for HGF-mediated malignant phenotypes (20,21,22). The present study shows that c-Src and Stat3 can also act as upstream regulators of HGF expression, and could lead to establishment of an HGF autocrine loop, signal amplification, and an invasive phenotype (Fig. 5). Evidence from our study supports the hypothesis that aberrant activation of c-Src and over-expression of Stat3, acquired during epithelial-mesenchymal transition of epithelial cells, could overcome the mechanisms repressing *HGF* transcription. In this model, Stat3 is activated by c-Src through phosphorylation of Tyr705 of Stat3, although concomitant phosphorylation of Ser727 of Stat3 by kinases such as Rac1 and p38 (29) is most likely required for optimal Stat3 activity. This activation process is distinct from mechanisms regulating basal levels of HGF expression, which involve Stat3-independent pathways (eg estrogen receptor or IL-6) (17). Since c-Src/Stat3-dependent activation of *HGF* transcription is preferentially associated with epithelial-mesenchymal transition, this signalling pathway may be an effective target for disruption of autocrine HGF loops and abrogation of breast cancer metastasis.

Future approaches will test the *in vivo* relevance of c-Src/Stat3 in regulation of HGF expression in an experimental metastasis model and in human breast carcinoma cells. The efficacy of pharmacological inhibitors of c-Src and Stat3 in blocking HGF expression and HGF/Met autocrine loop in breast carcinoma cells will also be assessed.

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APPENDICES: - attached

- I. Co-operative effect of c-Src tyrosine kinase and Stat3 in activation of hepatocyte growth factor expression in mammary carcinoma cells. W. Hung and B.E. Elliott. *J. Biol. Chem.* 276:12395-403, 2001.
- II. The role of hepatocyte growth factor/scatter factor in epithelial-mesenchymal transition and breast cancer. B.E. Elliott, W.L. Hung, A.H. Boag, and A.B. Tuck. *J. Physiol. Pharmacol.* 80:01-102, 2002.
- III. A novel activating function of c-Src and Stat3 on the *HGF* promoter in mammary carcinoma cells. J.E. Wojcik, R. Watering, E.A. Tremblay, K. Swan, C.R. Mueller, and B.E. Elliott. Submitted to *J. Biol. Chem.*, 2003.
- IV. A novel activating function of Stat3 on the *HGF* promoter in breast carcinoma cells. E.J. Wojcik, W.L. Hung, E.A. Tremblay, T.G. Wright, C.R. Mueller and B.E. Elliott. CBCRI Reasons for Hope Conference, October 25-27, 2003.
- V. Co-operative effect of c-Src and Stat3 in stimulating HGF expression and scattering in mammary carcinoma cells. B.E. Elliott. DOD Breast Cancer Research Program Meeting: Era of Hope. Orlando, Florida, September 25-29, 2002.

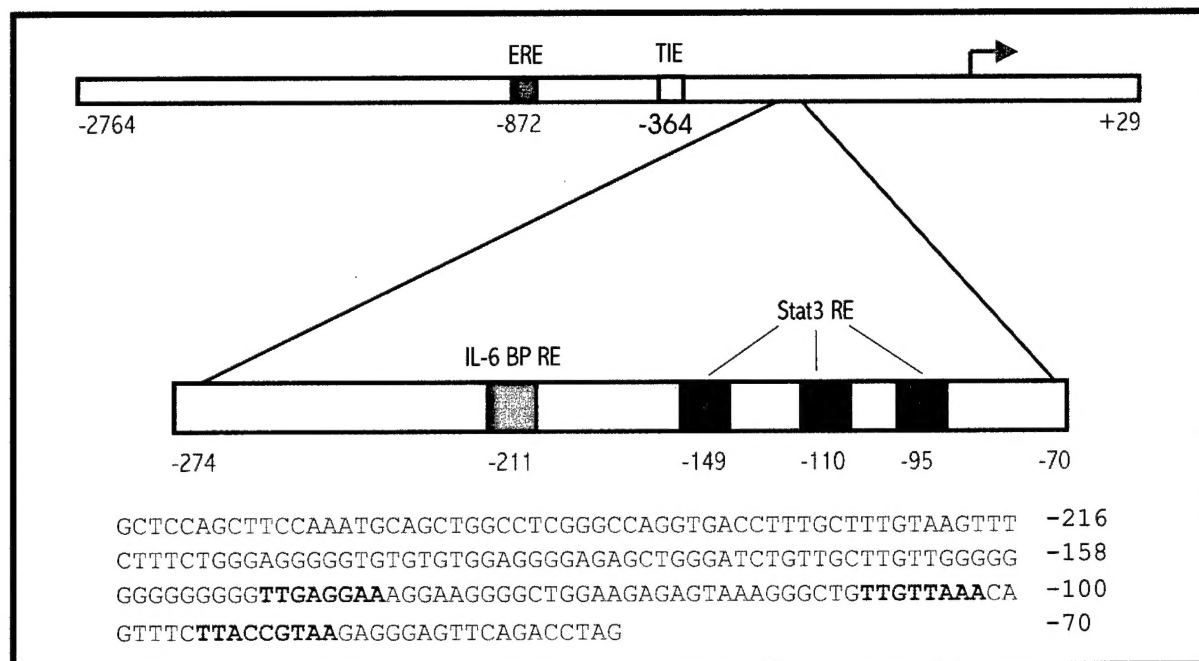


Fig. 1. Regulatory elements in the proximal region of the *HGF* promoter:

The relative positions of regulatory elements relevant to this proposal are shown. The c-Src/Stat3 responsive region is enlarged, showing the -149, -110 and -95 consensus Stat3 binding sites in blue. The nucleic acid sequence of this region is also shown, with the regulatory sequences highlighted in blue. The **IL-6 Binding Protein Responsive Element (IL-6 BP RE)** is shown in green, and the **Estrogen Responsive Element (ERE)** in red. The **TGF- β Inhibitory Element** is shown in yellow.

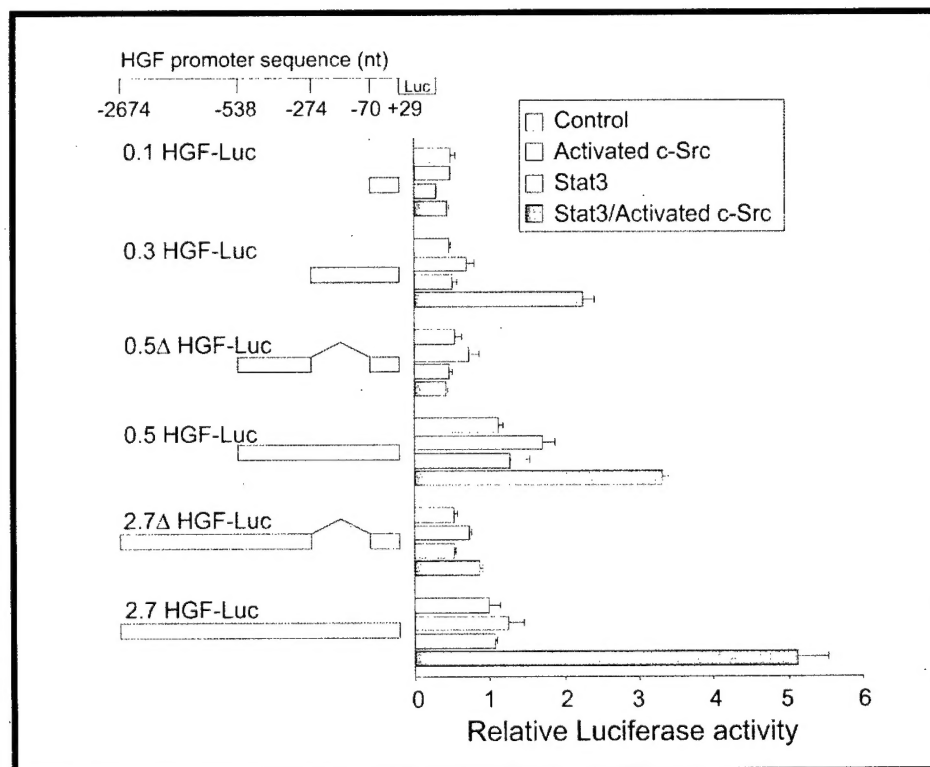


Fig. 2. The nt -274 to -70 region of the *HGF* promoter is required for c-Src/Stat3-induced *HGF* transcription in epithelial cells: The 2.7 kb *HGF*- firefly luciferase reporter (2.7 HGF-Luc), or reporter constructs containing various deletions of the *HGF* promoter were co-transfected into HC11 cells with activated c-Src, Stat3, c-Src/Stat3 or an empty vector (control). After 48 hours of incubation, cells were lysed and luciferase activity for each sample was determined and normalised. Values represent mean \pm SD of triplicate samples.

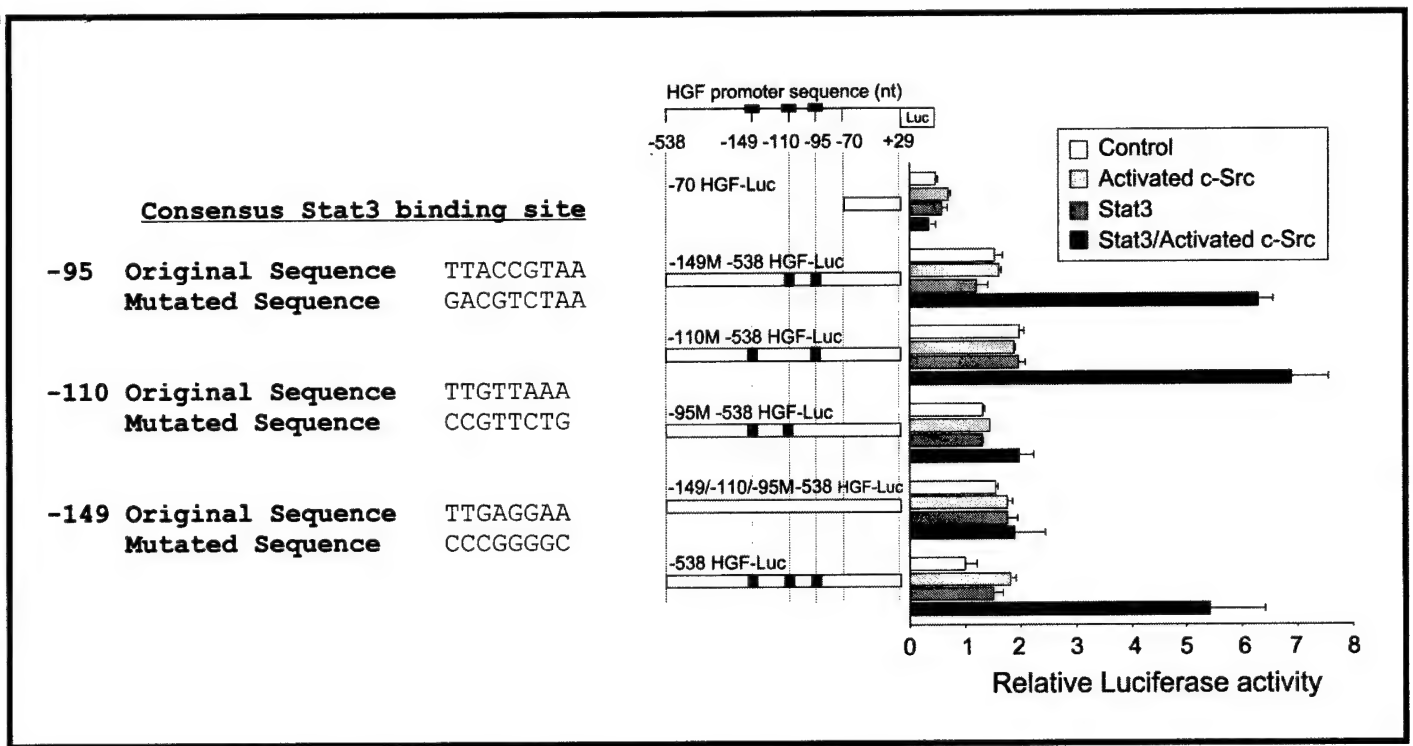


Fig. 3. Mutation of the -95 Stat3 binding site inhibits the responsiveness of HGF transcription to c-Src and Stat3 in HC11 cells. -538 HGF-Luc constructs mutated at the consensus Stat3 binding sites indicated were co-transfected into HC11 cells with activated c-Src, Stat3, c-Src/Stat3 or an empty vector (control), as indicated. -538 HGF-Luc and -70 HGF-Luc were used as positive and negative controls, respectively. After 48 hours of incubation, cells were lysed and luciferase activity for each sample was determined and normalised. Values represent mean \pm SD of eight samples.

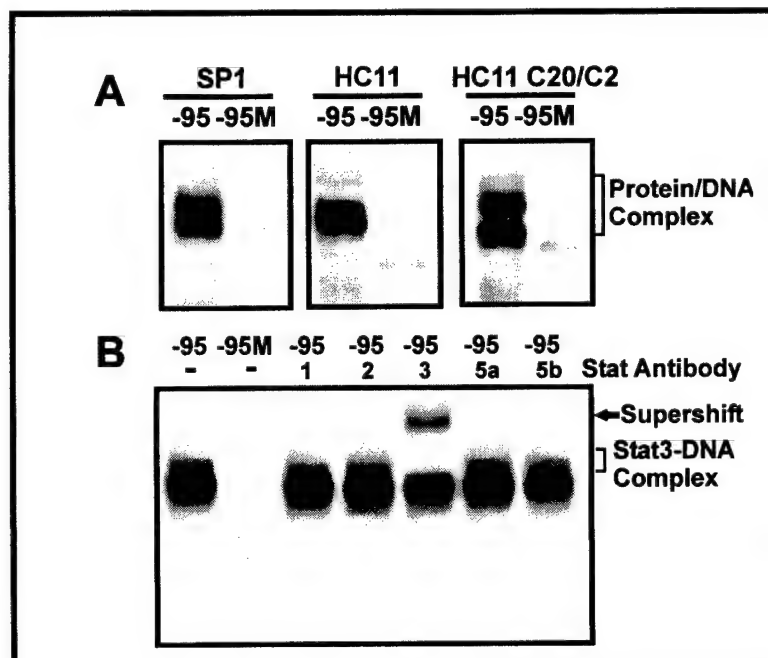


Fig. 4. Protein-DNA complexes form at the -95 consensus site.

Panel A) Equal protein amounts of nuclear extracts from SP1, HC11 and HC11 C20/C2 cells were used for EMSA binding studies with radiolabelled oligonucleotides containing either the 95 or the 95M sequence, as described in Materials and Methods. The arrow indicates the position of the protein-DNA complex.

Panel B) Nuclear extracts from HC11 C20/C2 cells were incubated with the indicated anti-Stat antibodies on ice for 30 min prior to EMSA analysis. Positions of the Stat3-DNA and the antibody-Stat3-DNA complexes are indicated.

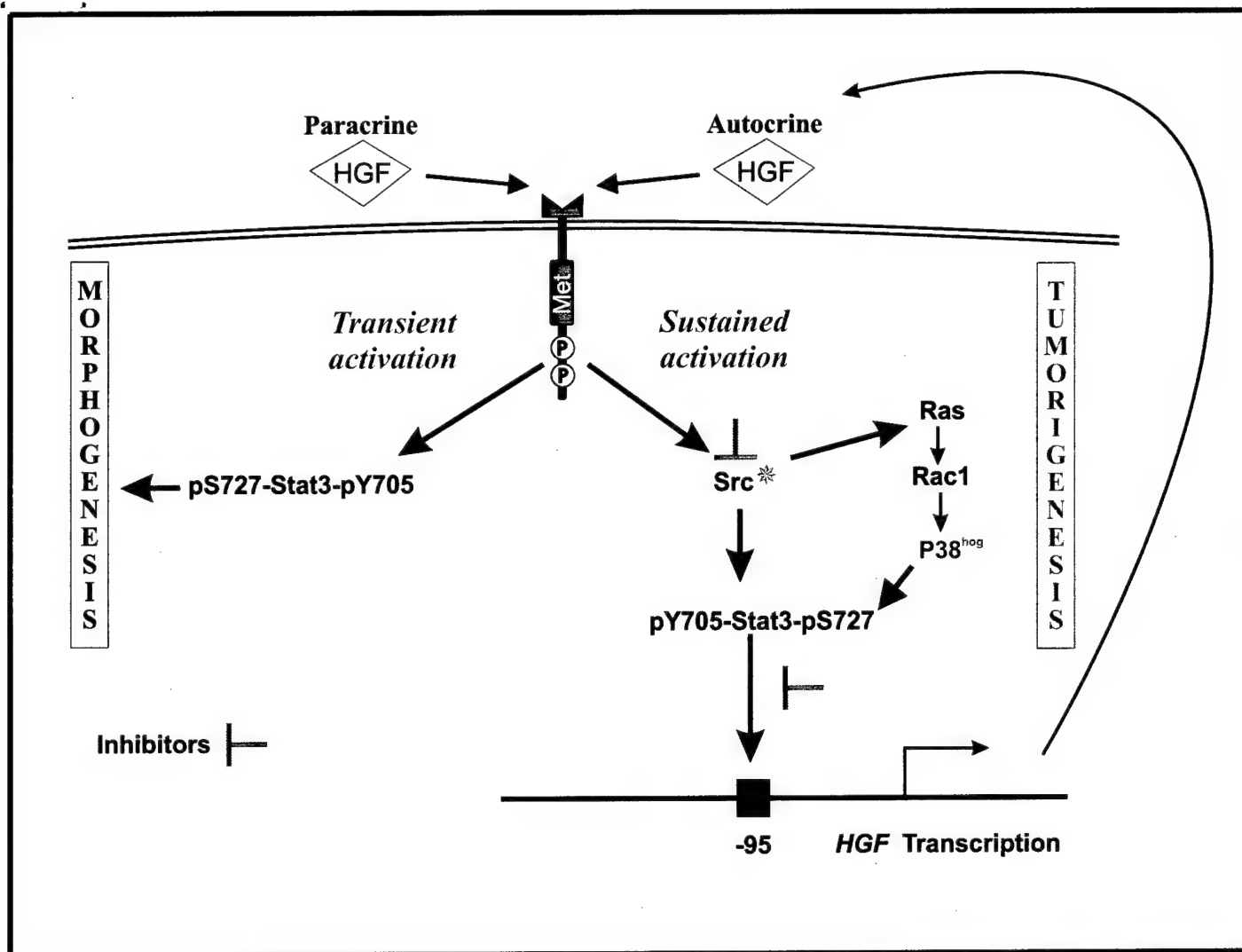


Fig. 5. Proposed regulation of HGF expression in breast cancer. Activated c-Src phosphorylates Stat3 at Tyr705, followed by homodimerization and translocation of Stat3 to the nucleus. c-Src could also activate Ras and Rac1/p38^{hog}, which phosphorylates Ser727 of Stat3. Fully activated Stat3 stimulates *HGF* transcription through binding to the -95 consensus site. HGF protein is synthesized, is secreted, and stimulates autocrine HGF/Met signalling. In contrast, transient activation of Stat3 promotes normal mammary morphogenesis independent of c-Src, and does not regulate *HGF* transcription. Inhibition of c-Src or Stat3 would be expected to block *HGF* transcription and protein expression.

Co-operative Effect of c-Src Tyrosine Kinase and Stat3 in Activation of Hepatocyte Growth Factor Expression in Mammary Carcinoma Cells*

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We have previously shown coexpression of hepatocyte growth factor (HGF) and its receptor Met in the invasive tumor front of human breast carcinomas. We have also demonstrated secretion of HGF, constitutive activation of Met, and increased invasion in a murine breast carcinoma cell line, SP1. These observations suggest the presence of an HGF autocrine loop in some breast carcinoma cells, which confers increased survival, growth, and invasiveness during tumor progression and metastasis. c-Src tyrosine kinase, which is critical in regulating the expression of many genes, is activated in SP1 carcinoma cells, as well as in most human breast cancers. We therefore examined the role of c-Src kinase in HGF expression in breast carcinoma cells. Expression of activated c-Src in SP1 cells increased transcription from the *HGF* promoter and expression of HGF mRNA and protein, while dominant negative c-Src had the opposite effect. Using deletion analysis, we showed that the region between –254 and –70 base pairs was required for c-Src responsiveness of the *HGF* promoter. This region contains two putative consensus sequences (at –110 and –149 base pairs) for the Stat3 transcription factor, which bind protein complexes containing Stat3 (but not Stat1, –5A, or –5B). Coexpression of activated c-Src and Stat3 synergistically induced strong *HGF* promoter activity in SP1 cells, as well as in a nonmalignant epithelial cell line, HC11 (HGF negative). c-Src kinase activity correspondingly increased the tyrosine 705 phosphorylation and DNA binding affinity of Stat3 (but not Stat1, –5A, or –5B). Collectively, our data indicate a cooperative effect of c-Src kinase and Stat3 in the activation of *HGF* transcription and protein expression in breast carcinoma cells. This process may be important in overriding the strong repression of *HGF* expression in nonmalignant epithelium, and thereby promote tumorigenesis.

Scatter factor, also known as hepatocyte growth factor

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(HGF),¹ is a multifunctional cytokine. Through binding to its receptor (Met), HGF can induce cell survival (1), growth (2), differentiation (3), and motility (4). It has been shown that both HGF and Met are essential for embryo development. Disruption of HGF expression in mice results in lethality in early development (5), while deletion of Met causes underdevelopment of limb buds (6). During development of the mammary gland, HGF is expressed by stromal cells, whereas epithelial cells express Met, but not HGF (7). Paracrine stimulation of normal breast epithelium with HGF, in cooperation with other growth factors (e.g. neuregulin), promotes branching morphogenesis (8). The tissue-specific suppression of HGF expression in normal epithelial cells provides a tightly controlled regulation of mammary ductal morphogenesis (9).

In contrast to normal breast epithelium, HGF and Met are frequently overexpressed in breast carcinomas (10–12) as well as many other cancer types (10, 11, 13, 14). This high level of HGF and Met expression has been identified as a possible independent predictor of poor survival in breast cancer patients (11). Our laboratory has previously shown that invasive human carcinoma cells coexpress HGF and Met, particularly at the migrating tumor front (12). We have also found that breast carcinoma cell lines frequently express HGF and Met, whereas most nonmalignant epithelial cell lines express Met, but not HGF.² Furthermore, overexpression of HGF or a constitutively active mutant form of Met (Tpr-Met) in transgenic mice (15, 16) or in transformed cell lines (17, 18) promotes tumorigenesis and metastasis. Together, these results suggest that establishment of an autocrine HGF loop and sustained activation of the Met signal transduction pathway in carcinoma cells may promote tumor progression. However, the mechanisms leading to aberrant expression of HGF in carcinoma cells are not known.

A number of signaling molecules, such as c-Src (19), Grb2/Ras (17), and phosphatidylinositol 3-kinase (1), have been shown to be part of the HGF/Met signaling pathway. Activation of Met through binding of HGF causes autophosphorylation of two specific tyrosine residues in the cytoplasmic tail of the receptor tyrosine kinase (20). These phosphorylated tyrosine residues act as multifunctional docking sites that bind the SH2 domain of specific cytoplasmic signaling molecules and causes their activation. The c-Src nonreceptor tyrosine kinase is expressed in many cell types, and its activity is increased in response to HGF and binding to Met (19). Increased activation of the tyrosine kinase c-Src occurs in many human cancer cells, and c-Src plays a critical role in breast cancer. Overexpression

¹ The abbreviations used are: HGF, hepatocyte growth factor; kb, kilobase pair(s); RT-PCR, reverse transcriptase-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); GUSB, β -galactosidase; PIPES, 1,4-piperazinediethanesulfonic acid.

² W. Hung, J. Gin, and B. Elliott, unpublished results.

of an activated form of c-Src in transgenic mice induces mammary hyperplasia (21). Furthermore, c-Src kinase is required in polyoma middle T-induced mammary tumorigenesis in transgenic mice (22). We have shown previously that c-Src kinase is constitutively activated in a mouse breast carcinoma cell line, SP1, which expresses both HGF and tyrosine-phosphorylated Met and which exhibits spontaneous invasion through matrigel (19, 23, 24). Furthermore, c-Src kinase activity is required for HGF-dependent cell motility and anchorage-independent growth of SP1 cells (19). Collectively, these findings indicate that c-Src kinase is an important requirement, but is not sufficient, for mammary tumorigenesis.

Activation of c-Src kinase can lead to increased expression of many genes, including growth factors such as vascular endothelial growth factor (25, 26) and parathyroid hormone-related peptide (27). We therefore hypothesized that elevated c-Src activity can promote increased HGF expression and the establishment of an HGF autocrine loop in SP1 cells. We observed that the c-Src tyrosine kinase inhibitor PP2 causes a 2-fold reduction in HGF transcription in SP1 cells. In addition, expression of a dominant negative mutant of c-Src (SRC-RF) in SP1 cells leads to similar levels of reduction in HGF mRNA and functional protein. Using deletion mutants of the HGF promoter, we have located a region (between -254 and -70) of the HGF promoter responsive to increased c-Src kinase activity in SP1 cells. This region contains two putative consensus binding sites for Stat3. Stat3 is a transcription factor originally described as the target of interferon receptors (28), but recent reports have indicated that Stat3 can be activated by c-Src kinase via platelet-derived growth factor (29) and HGF receptors (30), and is important in mammary differentiation (30). We therefore examined the role of Stat3 in c-Src-dependent regulation of HGF transcription. The results indicate that while expression of Stat3 alone increased HGF promoter activity, simultaneous expression of Stat3 and activated c-Src led to strong cooperative activation of HGF transcription in both non-malignant epithelial and carcinoma cells. Expression of mutant c-Src kinases in breast carcinoma cells altered both the tyrosine phosphorylation status and DNA binding activity of Stat3. While activated c-Src induced Stat3 tyrosine phosphorylation and DNA binding activity, a dominant negative mutant of c-Src reduced tyrosine phosphorylation and DNA binding. Together these data suggest that c-Src kinase and Stat3 act cooperatively in the activation of HGF expression in breast carcinoma cells, and may be important in overriding the strong repression of HGF expression in nonmalignant epithelial cells.

MATERIALS AND METHODS

Antibodies and Reagents—Rabbit anti-c-Src IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody EC10 against chicken c-Src was a gift from Dr. S. Parsons. Rabbit anti-sheep IgG conjugated with horseradish peroxidase was from Jackson ImmunoResearch Laboratories (West Grove, PA). Sheep anti-HGF IgG was a gift from Genentech (San Francisco, CA). Rabbit anti-HGF antibody was generated against recombinant glutathione S-transferase-HGF-(1-120) protein in our laboratory at Queen's University, this antibody recognizes only the N-terminal portion of HGF (data not shown). Anti-Stat1, -Stat3, -Stat5A, and -Stat5B and anti-phospho-Stat3 (Y705) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). c-Src family kinase inhibitor PP2 was obtained from Calbiochem (La Jolla, CA).

Plasmid Construction—c-Src expression plasmids were constructed by subcloning activated (Y527F) and dominant negative (K295R,Y527F) chicken c-src cDNAs (gift from Drs. J. Brugge and D. Shalloway) into the EcoRI site of DNA polymerase I (Klenow fragment)-treated pBabePuro plasmid to generate pBabe Y527F and pBabe Src-RF. A reporter construct containing the full-length HGF promoter region fused to luciferase (2.7 HGF-luc) was constructed by ligating the HindIII/XbaI fragment (treated with DNA polymerase I (Klenow fragment)) of 2.8 HGF-CAT (gift from Dr. R. Zarnegar) into the HindIII site

of pGL2-Basic (Promega), also treated with DNA polymerase I (Klenow fragment). Further deletions were constructed by cutting 2.7 HGF-luc with *SmaI*, *SacI*, and *BglII*, followed by re-ligation to generate 0.5 HGF-luc, 0.3 HGF-luc, and 0.1 HGF-luc, respectively. The 1.2 HGF-luc was constructed by ligating the 1.4-kb *SaI* fragment from 2.7 HGF-luc into the *XhoI* site of pGL2-Basic. An internal deletion mutant 0.5Δ HGF-luc was constructed by digestion of 0.5 HGF-luc with *PvuII*/*BglII* and treatment with DNA polymerase I (Klenow fragment) before re-ligation. The Δ1 HGF-luc was constructed by ligating the *SmaI* fragment of 2.7 HGF-luc into the same site of 0.5Δ HGF-luc. The Δ2 HGF-luc was constructed by ligating the *SmaI* fragment of 2.7 HGF-luc into 0.8 HGF-luc. The ΔΔ HGF-luc was made by ligating the *SmaI* fragment of Δ2 HGF-luc into the same site of 0.5Δ HGF-luc. For normalization of transfection efficiency of each sample, pSG5βgal (a gift from Dr. M. Petkovich) or pCHCβgal (a gift from Dr. F. Kern) (31), which expresses β-galactosidase under the control of SV40 and cytomegalovirus promoters, respectively, was used.

Tissue Culture and Cell Lines—The SP1 tumor cell line is derived from a spontaneous poorly metastatic murine mammary intraductal adenocarcinoma, and expresses both HGF and Met. The characterization of the SP1 cell line has been described previously (19, 23, 24). Maintenance medium for SP1 cells was RPMI 1640 supplemented with 7% fetal bovine serum. HC11 is a mammary epithelial cell line (32) and was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, insulin (5 μg/ml), and epidermal growth factor (10 ng/ml).

Cell Transfection—All transfections were carried out with LipofectAMINE Plus reagent (Canadian Life Technology, Burlington, ON, Canada) according to manufacturer's instructions. Cells (15,000) were seeded in a 24-well plate and transfected with 0.4 μg of reporter plasmid, 0.1 μg of pSG5βgal, and up to 0.4 μg of expression plasmids (such as c-Src) as indicated. After 48 h, transfected cells were harvested and lysed. One-fifth of the cell lysate was used to assay for β-galactosidase activity, an equal amount of lysate was used for a luciferase assay using PharMingen Luciferase Substrates (BD PharMingen, Mississauga, ON). Luciferase activity was measured using a luminometer with wavelength at 562 nm. Luciferase activity of each sample was normalized to the corresponding β-galactosidase activity. For immunoprecipitation and *in vitro* c-Src kinase assays, 2.5×10^5 cells were seeded in a 100-mm tissue culture plate and transfected with 4 μg of reporter plasmid, 1 μg of pSG5-β-galactosidase, and up to 4 μg of expression plasmids as indicated. One-tenth of the cells was used for a luciferase assay, and the remaining cells were lysed and used for immunoprecipitation.

To obtain stably transfected cells, SP1 cells were plated at 70% confluence in 60-mm plates and transfected with 2 μg of plasmids expressing various mutants of c-Src. Puromycin (2 μg/ml, Sigma, Oakville, ON) was added to cells 24 h following transfection, and was maintained until all cells in the mock transfection were killed. Puromycin-resistant cells were then collected and used as pooled cell lines. Expression and activity of c-Src mutants in transfected cells were checked using Western blotting analysis and a c-Src kinase assay. Total c-Src protein was immunoprecipitated with an excess amount of anti-c-Src (pan) antibody to maximize the amount of antibody-protein complex formed. We have previously found that these c-Src mutants are quite effective, and that relatively small levels of expression can result in significant phenotypes (19).

RNA Isolation and RT-PCR—Cells grown to 80% confluence on a 100-mm dish were washed and lysed with TriZol reagent (Canadian Life Technology). Phase separation was achieved by addition of chloroform and centrifugation at top speed in a microcentrifuge for 10 min. Aqueous phase containing total RNA was removed to a new tube and precipitated with an equal volume of isopropyl alcohol for 10 min at room temperature. The RNA pellet was recovered by centrifugation and washed with 70% ethanol. After brief drying, the RNA pellet was resuspended in diethyl pyrocarbonate-treated water. RNA concentration was determined by spectrophotometry. An aliquot (1 μg) of total RNA was used for reverse transcription with avian myeloblastosis reverse transcriptase at 42 °C for 15 min. One-tenth of the reaction was used in PCR analysis with end-labeled oligonucleotides specific for HGF (5'-TGTCGCCATCCCTATGCAG-3' and 5'-GGAGTCACAAGTCT-TCAACT-3') and β-glucuronidase (*GUSB*) sequences, as previously described (33). The PCR reaction conditions were 2 min at 95 °C, followed by 25 cycles of 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C, and a final cycle of 10 min at 72 °C. The reaction was then analyzed on a 2% agarose gel by electrophoresis. The bands corresponding to the HGF and *GUSB* products were excised and the amount of radioactivity was determined by scintillation counting.

Copper Affinity Column Chromatography—Conditioned media were collected and HGF was partially purified using copper (II) affinity column chromatography, as described previously (34). Cells were grown to 80% confluence. The cell monolayer was washed with fresh Dulbecco's modified Eagle's medium and incubated in serum-free Dulbecco's modified Eagle's medium for 24 h. Conditioned media were collected, and cell debris was removed by centrifugation. Conditioned medium (10 ml) from each cell line was then loaded onto a copper (II) affinity column. The copper (II) affinity column was prepared by chelating Cu^{2+} ions on a 1-ml HiTrap Chelating column (Amersham Pharmacia Biotech, Baie d'Urfe, PQ), and equilibrated with equilibration buffer (20 mM sodium phosphate, pH 7.2, 1 M NaCl, 1 mM imidazole). The conditioned medium was recycled through the column 5 times to ensure binding of all HGF proteins, and the column was washed thoroughly with 15 volumes of equilibration buffer. HGF protein was eluted from the column with equilibration buffer containing 80 mM imidazole at a flow rate of 1 ml/min. Fractions of 1 ml each were collected; previous experiments have determined that essentially all HGF was eluted in fraction 2 (Ref. 34 and data not shown). The fraction containing HGF was concentrated by centrifugation with Microcon centrifugal filter devices (Millipore Corp., Bedford, MA) with a 10-kDa molecular mass cut off. The samples were analyzed on a denaturing 10% SDS-PAGE gel, followed by Western blotting with anti-HGF antibody.

Immunoprecipitation, Western Blotting Analysis, and c-Src Kinase Assay—Cells were grown to confluence and treated as indicated. After three washes with cold phosphate-buffered saline, cells were lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na_2VO_4 , 50 mM NaF, 2 mM EGTA, 2 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation and protein concentrations were determined by a bicinchoninic acid protein assay (Pierce, Rockford, IL). For immunoprecipitation, equal amounts of lysate were incubated with the indicated antibodies at 4 °C for 2 h or overnight. Immunoprecipitates were collected on protein A-Sepharose (Amersham Pharmacia Biotech), washed three times with lysis buffer, separated by SDS-PAGE gel, and transferred to a nitrocellulose membrane. Western blotting analysis was performed as described previously (19).

In vitro c-Src kinase assays were performed as described previously (19). Briefly, each lysate was immunoprecipitated with anti-c-Src IgG (Santa Cruz Biotechnology) as described above. One-half of each immunoprecipitate was subject to SDS-PAGE under nondenaturing conditions and Western blot analysis to confirm the amount of c-Src protein present. The other half of each immunoprecipitate was assayed for c-Src kinase activity by incubating with 10 μl of reaction buffer (20 mM PIPES, pH 7.0, 10 mM MnCl_2 , 10 μM Na_3VO_4), 1.4 μg of freshly prepared acid-denatured enolase (Sigma), and 10 μCi of [γ - ^{32}P]ATP. After a 10-min incubation at 30 °C, reactions were terminated by the addition of 2 \times SDS sample buffer, and samples were subjected to 8% SDS-PAGE. Serine and threonine phosphorylations were hydrolyzed by incubating the acrylamide gel in 1 M KOH at 45 °C for 30 min, followed by fixing in 45% MeOH and 10% acetic acid for 30 min at room temperature. The gel was dried under vacuum. Autoradiograms were produced and analyzed with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Oligonucleotides and Probe Labeling—Oligonucleotides used for electrophoretic mobility shift assay binding were Stat3-110F (5'-GGGCT-GTTGTAAACAGT-3'), Stat3-110R (5'-AGAACTGTTAACAACAG-3'), Stat3-149F (5'-GGGGTTGAGGAAAGGAAG-3'), and Stat3-149R (5'-CCCCTTCCTTTCCTCAAC-3'). Complementary oligonucleotides were annealed by boiling equal molar amounts of each oligonucleotide for 10 min and then cooling slowly to room temperature. The annealed oligonucleotides (20 pmol) were labeled by a filling-in reaction with Klenow enzyme and [α - ^{32}P]dCTP.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared as described previously (35). Briefly, 10^7 cells were washed once with phosphate-buffered saline before resuspension in cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate). Cells were allowed to swell on ice for 10 min before lysis by brief vortexing. Nuclei were pelleted and resuspended in buffer C (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate). High salt extraction was performed by incubation on ice for 30 min in buffer C and centrifugation at 4 °C. The protein content of the supernatant (nuclear extract) was determined using a Bradford protein assay (Bio-Rad, Mississauga, ON).

Electrophoretic mobility shift assays were performed as described by

Mohan *et al.* (36). Briefly the binding reaction was performed by incubating 5 μg of nuclear extracts with 0.1 pmol of ^{32}P -labeled oligonucleotide probe in the presence of binding buffer (10 mM HEPES, pH 7.9, 60 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 9% glycerol, and 4 μg of poly(dI-dC) (Amersham Pharmacia Biotech). Binding was allowed to proceed at room temperature for 10 min before analysis on 5% nondenaturing PAGE gel in Tris glycine buffer (40 mM Tris-HCl, pH 8.4, 266 mM glycine). When unlabeled oligonucleotides were added, 10-fold molar excess was included in the binding reaction. For supershifting experiments, nuclear extracts were incubated with 2 μg of the indicated antibody at room temperature for 20 min prior to the binding reaction. After electrophoresis, the gel was fixed in 7% acetic acid, 40% methanol for 30 min, and dried under vacuum. The gel was then exposed to a PhosphorImager screen, and analyzed using a Storm PhosphorImager.

RESULTS

Inhibition of Activity of c-Src Family Kinases Impairs HGF mRNA Expression—To study the regulation of HGF expression in breast carcinoma cells, we used the mouse mammary carcinoma cell line SP1, which coexpresses HGF and tyrosine-phosphorylated Met (23). Semi-quantitative RT-PCR was performed to determine the levels of HGF mRNA in SP1 cells. We first examined the dose-dependent effect of an inhibitor of c-Src family kinases, PP2 (37). Total RNA was isolated from SP1 cells treated with different concentrations of PP2 and used for cDNA synthesis by reverse transcription. Relative HGF mRNA levels were determined by RT-PCR with HGF-specific primers, and each sample was normalized to the expression of a housekeeping gene β -glucuronidase (*GUSB*) (33). The results showed that the PP2 inhibitor reduced HGF mRNA expression in a dose-dependent manner up to 40% of untreated cells (Fig. 1A). In addition, we examined the level of transcription of the *HGF* gene using a reporter plasmid. A plasmid containing a 2.7-kb fragment 5' of the *HGF* transcriptional start site ligated to the firefly luciferase gene was transiently transfected into SP1 cells. Bell *et al.* (38) have previously shown that this 2.7-kb fragment of the *HGF* promoter contains all the necessary sequence to direct HGF expression and mimics the expression pattern of the endogenous *HGF* gene in transgenic mice. Following transfection, these cells were treated with different concentrations of the PP2 inhibitor under conditions used in Fig. 1A. After a 24-h incubation, the cells were lysed and luciferase activity in each sample was determined and compared with control cells. The results show a similar dose-dependent reduction of *HGF* transcription following PP2 treatment (Fig. 1B). These findings suggest that the activity of c-Src kinase family members is important in the regulation of *HGF* transcription and mRNA expression.

c-Src Kinase Activity Regulates HGF Expression at Both mRNA and Protein Levels—We further investigated the role of c-Src tyrosine kinase in HGF expression by transfecting chicken c-Src mutants (SRC-Y527F and SRC-RF) with altered kinase activity into SP1 cells. The SRC-Y527F mutant contains a phenylalanine substitution at tyrosine 527 which results in constitutive kinase activity (39, 40). The SRC-RF mutant contains a double substitution at tyrosine 527 to phenylalanine and at lysine 295 to arginine, which produces a dominant negative phenotype (26). We have previously shown that expression of a similar dominant negative form of murine c-Src in SP1 cells reduces endogenous c-Src kinase activity and also impairs anchorage-independent growth in soft agar (19). As predicted, expression of the dominant negative form of chicken c-Src (SRC-RF) also decreased total c-Src kinase activity in SP1 cells, when compared with untransfected cells (Fig. 2, top panel). In addition, expression of the activated form of c-Src (SRC-Y527F) dramatically increased total c-Src kinase activity in SP1 cells. Expression of the chicken c-Src mutants was detected by an antibody (EC10) specific for avian c-Src (Fig. 2, bottom panel).

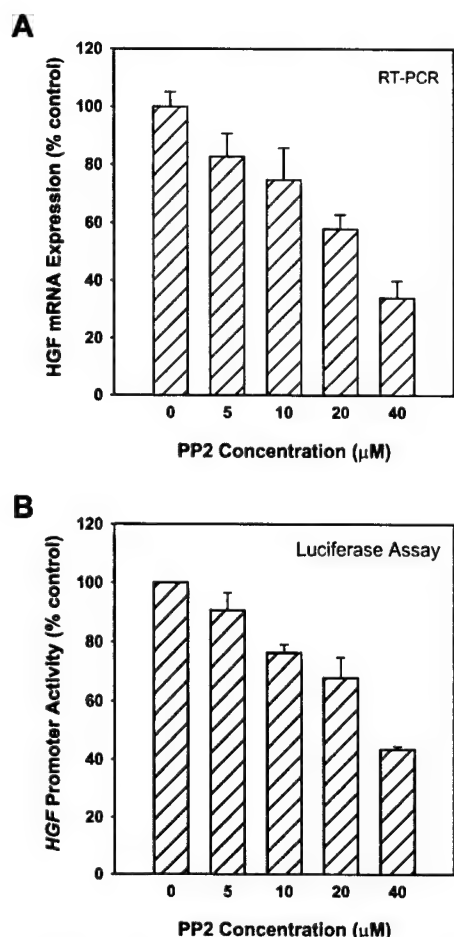


FIG. 1. Treatment with the c-Src family kinase inhibitor PP2 decreases HGF mRNA level and transcription. Panel A, pre-starved SP1 cells were incubated with the Src family kinase inhibitor PP2 at the concentrations indicated. After 24 h, cells were lysed and total RNA was extracted. The amount of HGF mRNA in each sample was quantitated using RT-PCR with HGF-specific primers and primers for GUSB (see "Materials and Methods"). The amount of HGF mRNA was normalized to GUSB mRNA, and the level of HGF mRNA expression in each group was expressed as a percentage of that in untreated (control) cells. Values represent the mean of two experiments \pm range. Panel B, SP1 cells were transfected with a reporter plasmid containing the 2.7-kb fragment of the HGF promoter driving expression of the luciferase gene (2.7 HGF-luc). A β -galactosidase expression plasmid was co-transfected in each group for normalization to account for differences in transfection efficiency. After 24 h of incubation, PP2 was added at the concentrations indicated, and the cells were incubated for an additional 24 h, lysed, and assayed for luciferase activity. Luciferase activity of each sample was expressed as a percentage of control (untreated) cells. Values represent the mean \pm S.D. of triplicate samples. The experiment was done twice with similar results.

To assess the effect of c-Src kinase activity on HGF mRNA expression, RT-PCR analysis was carried out on RNA extracted from SP1 cells expressing the different c-Src mutants, or treated with the PP2 inhibitor (Fig. 3A). Expression of the dominant negative SRC-RF mutant or treatment with PP2 reduced the HGF mRNA level in SP1 cells by \sim 60%. Conversely, expression of the constitutively active SRC-Y527F mutant increased HGF mRNA expression by about 2-fold. In a parallel approach, the level of secreted HGF protein was compared in conditioned media collected from the same cells and under the same conditions described in Fig. 3A. Our laboratory has previously shown that HGF is a Cu(II)-binding protein, which can be purified with high recovery from conditioned media with copper (II) affinity chromatography (34) and analyzed on a denaturing SDS-PAGE gel (Fig. 3B). Using this

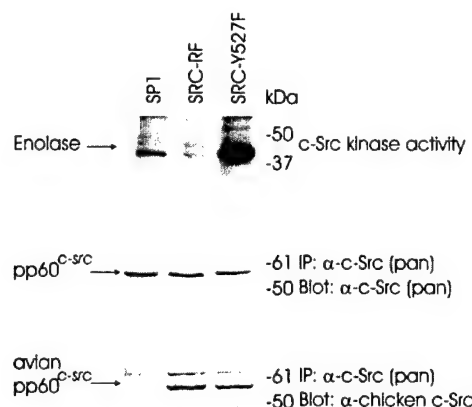


FIG. 2. Ectopic expression of c-Src kinase mutants in SP1 cells. SP1 cells were transfected with expression vectors containing activated c-Src (SRC-Y527F) or dominant negative c-Src (SRC-RF) or an empty expression vector (SP1). After 48 h, cells were lysed. Equal amounts of the cell lysates were immunoprecipitated with anti-c-Src (pan) antibody at excess antibody concentration. Half of the immunoprecipitates was used to detect c-Src kinase activity using enolase as a substrate (top panel). The other half was subjected to Western blotting with anti-Src (pan) antibody to confirm equal amounts of total c-Src protein in the immunoprecipitates (middle panel), and then reprobed with monoclonal anti-chicken c-Src (EC10) antibody to detect the relative level of ectopic expression of each c-Src mutant (bottom panel). The amount of chicken c-Src compared with total c-Src may be relatively low, and cannot be directly inferred from these results, since different antibodies and exposure times were used for each Western blot.

method, we showed that expression of the dominant negative SRC-RF mutant or treatment with PP2 significantly decreased the amount of HGF protein secreted by SP1 cells. In contrast, expression of activated c-Src (SRC-Y527F) increased the amount of secreted HGF protein. Together these data suggest that HGF expression (both at the mRNA and protein levels) is regulated by c-Src kinase activity.

c-Src Kinase Activity Induces HGF Expression through a Specific cis-Acting Region on the HGF Promoter—To determine the effect of c-Src kinase mutants on HGF promoter activity, we constructed a series of reporter plasmids with the luciferase gene linked to different fragments of the 2.7-kb region 5' of the HGF transcriptional start site (Fig. 4B). These reporter constructs were co-transfected into SP1 cells with a control vector, or vectors expressing the SRC-Y527F or SRC-RF mutants of chicken c-Src kinase, and luciferase activity of the transfected cells was compared (Fig. 4A). The results show that expression of activated c-Src increased up to 2-fold the activity of the 2.7-kb HGF promoter, whereas dominant negative c-Src had the opposite effect. Deletions of up to -538 bp (0.5 Δ HGF) had no significant effect on the c-Src dependent activity of the HGF promoter, although some fluctuations in basal activity of the promoter were apparent. A further deletion of -273 bp (0.3 HGF-luc) significantly reduced the basal HGF promoter activity, while some c-Src dependent activity remained. The remaining c-Src kinase responsiveness was eliminated when all but 72 bp (0.1 HGF-luc) of the HGF promoter was removed. This suggests that a cis-acting element responsive to c-Src kinase activity is located within -273 and -70 bp of the HGF promoter. An internal deletion construct lacking the -70 to -254 -bp region (named 0.5 Δ HGF-luc) was used to confirm the c-Src responsiveness of this region. As predicted, the 0.5 Δ HGF-luc reporter did not respond to expression of SRC-Y527F, although basal activity remained. A similar pattern of repression of the luciferase activity among all the HGF promoter deletion mutants used was seen when dominant negative c-Src (SRC-RF) was coexpressed with the HGF-luc constructs.

To confirm the importance of the regions of the promoter

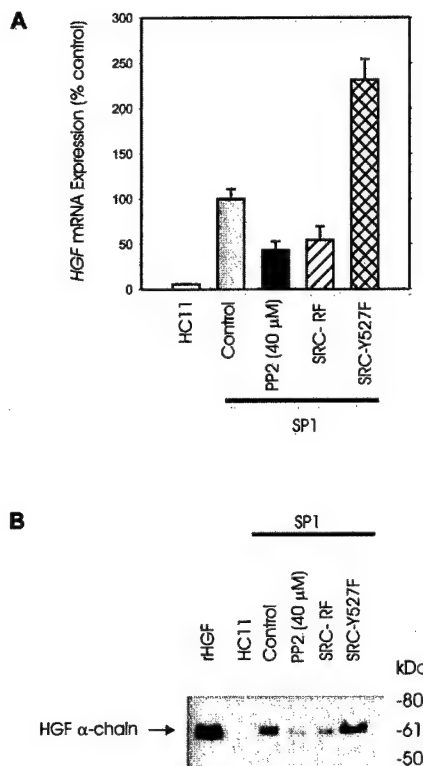


FIG. 3. c-Src kinase activity modulates HGF mRNA and protein levels in SP1 cells. *Panel A*, SP1 cells transfected with dominant negative Src (SRC-RF) or activated Src (SRC-Y527F) or empty vector (control) were prestarved overnight. PP2 (40 μM) was added to one plate of SP1 cells and incubated for an additional 24 h. A nonmalignant breast epithelial cell line HC11 was used as a negative control. Total RNA was isolated, and the amount of HGF mRNA in each sample was quantitated using RT-PCR and normalized to GUSB mRNA as described in the legend to Fig. 1. The level of HGF mRNA expression in each group was expressed as a percentage of that in untreated (control) cells. Values represent the mean of two experiments \pm range. *Panel B*, serum-free conditioned media were collected for 24 h from HC11 cells, PP2-treated SP1 cells, and SP1 cells transfected as in *Panel A*. HGF protein from the conditioned media was purified using copper (II) affinity chromatography (34). The fraction containing HGF protein was concentrated in Microcon concentrators and subjected to denaturing SDS-PAGE. Recombinant HGF (100 ng) was included in one lane as a control. After electrophoresis, the proteins were transferred onto nitrocellulose and the blot was probed with anti-HGF antibody. Immunoreactive bands were revealed using Enhanced Chemiluminescence kit.

responsive to activated c-Src, several internal deletion mutants were constructed. Full-length reporter constructs missing -273 to -70 bp ($\Delta 1$), -1231 to -755 bp ($\Delta 2$), or both regions ($\Delta\Delta$) of the *HGF* promoter were transfected into SP1 cells in the presence or absence of the SRC-Y527F and SRC-RF mutants (Fig. 4A). As predicted, $\Delta 1$ and $\Delta\Delta$ deletion mutants exhibited neither induction nor repression of *HGF* promoter activity when activated c-Src or dominant negative c-Src was expressed, respectively. In contrast, the $\Delta 2$ mutant showed strong induction of *HGF* promoter activity corresponding to expression of the activated SRC-Y527F mutant, and strong repression of *HGF* promoter activity when the SRC-RF mutant was expressed. This finding shows that only the region between -254 and -70 bp of the *HGF* promoter is important for c-Src responsiveness of *HGF* expression in SP1 cells. We will refer to this region as the c-Src responsive region.

Stat3 Activates HGF Transcription in Cooperation with Activated c-Src—Examination of the c-Src responsive region of the *HGF* promoter revealed several Stat3-binding sites. This consensus sequence is highly conserved among mouse, rat, and human (100% identity), while this conservation is lost in re-

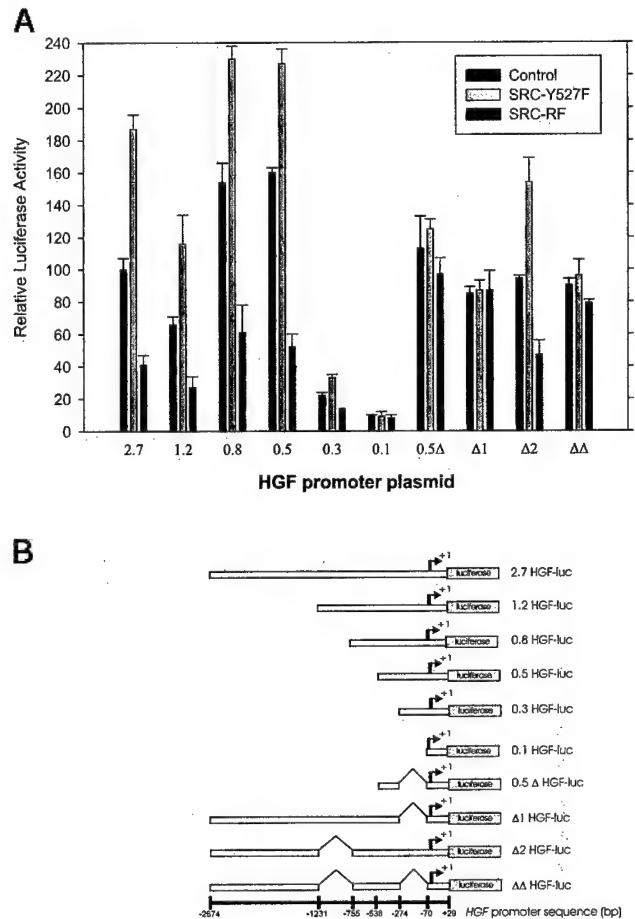
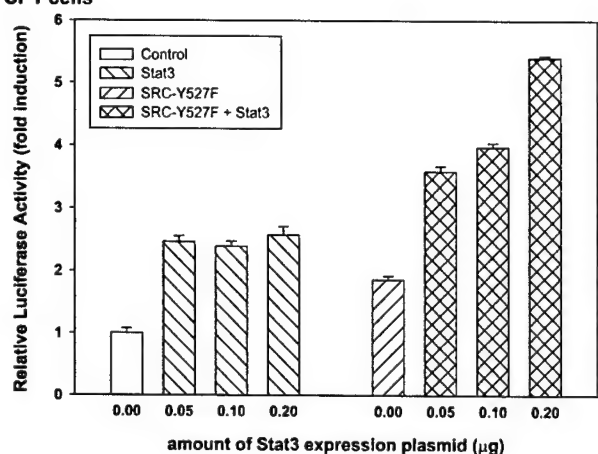


FIG. 4. c-Src kinase responsiveness of HGF transcription requires the -254 to -70 bp region of the HGF promoter. *Panel A*, the 2.7-kb HGF-luciferase reporter (2.7 HGF-luc), or reporter constructs containing various deletions of the HGF promoter (see *Panel B*), were co-transfected into SP1 cells with activated c-Src (SRC-Y527F), dominant negative c-Src (SRC-RF), or an empty expression vector (control). Luciferase activity of each sample was determined, and normalized to the empty vector control value within each group as described in the legend to Fig. 1B. Values represent mean \pm S.D. of triplicate samples. The experiments were done three times using two different preparations of plasmid DNA with similar results. *Panel B*, schematic representation of the wild-type *HGF* reporter construct and the corresponding internal deletion mutants used in *Panel A* is shown. The name of each construct refers to the full-length (2.7 kb) or truncated promoter sequences (1.2, 0.8, 0.5, 0.3, and 0.1 kb) upstream of the transcriptional start site (indicated by arrow). In addition, constructs containing the 0.5-kb sequence with an internal deletion of the region between -254 and -70 (0.5Δ), or the full-length sequence containing internal deletion of regions between -254 and -70 ($\Delta 1$), -1231 and -755 ($\Delta 2$), or both ($\Delta\Delta$) were used.

gions upstream of -500 bp of the *HGF* promoter (41). Since Stat3 activation by Src induces specific gene expression and is required for cell transformation (42, 43), we examined whether expression of Stat3 in the presence or absence of the activated c-Src mutant (SRC-Y527F) has any effect on *HGF* promoter activity. A reporter plasmid containing the -2.7-kb full-length *HGF* promoter was co-transfected with a constant amount of the SRC-Y527F, and varying amounts of Stat3, expression plasmids. Expression of activated c-Src (SRC-Y527F) alone increased *HGF* transcription by about 2-fold (Fig. 5A). Likewise, expression of Stat3 alone increased *HGF* transcription by about 2-fold, and maintained a plateau value with even 0.05 μg of plasmid DNA. However, in cells coexpressing both the activated c-Src mutant and increasing amounts of Stat3, *HGF* transcription increased up to 5-fold. This result indicates that there is a cooperative effect between c-Src kinase activity and

A. SP1 cells



B. HC11 cells

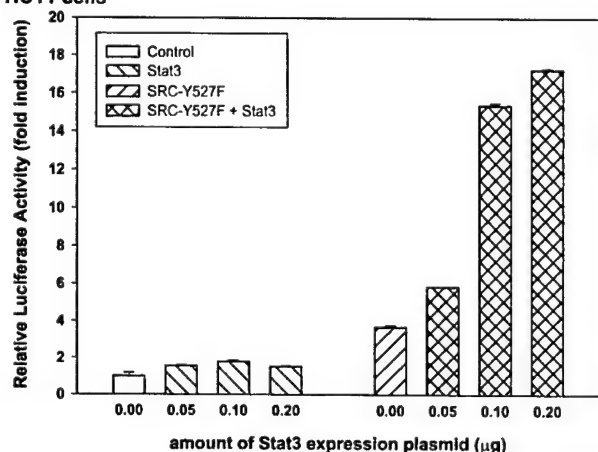


FIG. 5. Stat3 induces HGF transcription in cooperation with activated c-Src. SP1 carcinoma cells (Panel A) and HC11 mammary epithelial cells (Panel B) were co-transfected with the 2.7 HGF-luc reporter and activated c-Src (SRC-Y527F) or an empty vector (control), in combination with varying amounts of Stat3. Luciferase activity was determined and expressed as a percentage of that in control cells as described in the legend to Fig. 1B. Values represent the mean \pm S.D. of triplicate samples. The experiment was done twice with similar results.

Stat3 protein in the regulation of *HGF* transcription.

The nonmalignant mammary epithelial cell line, HC11, shows at least a 15-fold lower level of *HGF* transcription and no detectable *HGF* protein, compared with SP1 carcinoma cells (data not shown). We therefore determined whether coexpression of c-Src and Stat3 can activate *HGF* transcription in HC11 cells. Expression of activated c-Src induced expression by about 4-fold (Fig. 5B). In contrast to SP1 cells, expression of Stat3 alone in HC11 cells did not significantly induce *HGF* transcription. However, when activated c-Src and Stat3 were coexpressed, *HGF* transcription was synergistically induced up to 17-fold. Similarly, two clones of HC11 cells, stably transfected with Stat3 followed by transient expression of activated c-Src, showed up to a 20-fold increase in *HGF* promoter activity (data not shown). Together, these results suggest that increased c-Src kinase activity and Stat3 expression can override the repression of *HGF* transcription in nonmalignant mammary epithelial cells.

To determine whether the c-Src responsive region of the *HGF* promoter is involved in the observed cooperative effect between c-Src and Stat3, the transcriptional activity of a mutant *HGF* reporter lacking the c-Src responsive region ($\Delta 1$ HGF-luc) was compared with that of the full-length (2.7 HGF-luc) *HGF* reporter. Each reporter construct was transfected into SP1 cells alone, or in combination with Stat3, and the

activated c-Src (SRC-Y527F) mutant, expression plasmids. Expression of the activated c-Src mutant induced activation of the full-length *HGF* promoter, but not of the deletion mutant ($\Delta 1$ HGF-luc) (Fig. 6). Similarly, Stat3 expression increased the activity of the full-length *HGF* promoter, and only marginally affected that of the deletion mutant ($\Delta 1$ HGF-luc), this result suggests that Stat3 activates the *HGF* promoter. The level of induction due to Stat3 expression is even higher than that due to activated c-Src alone. This effect is probably due to a limiting amount of endogenous Stat3 in SP1 cells. When both Stat3 and activated c-Src were coexpressed, *HGF* promoter activity in the full-length construct was strongly induced, this effect was not seen in the deletion mutant ($\Delta 1$ HGF-luc). These results show a cooperative effect between Stat3 and activated c-Src in the induction of *HGF* transcription, and imply the presence of specific Stat3-binding sites on the *HGF* promoter.

c-Src Kinase Regulates Tyrosine 705 Phosphorylation and DNA Binding Activity of Stat3—Previous reports have found that c-Src activates Stat3 by inducing tyrosine phosphorylation of Stat3 and increasing its DNA binding affinity (42, 44). We therefore examined the effect of c-Src kinase activity on Stat3 tyrosine 705 phosphorylation in SP1 cells. We found that expression of activated c-Src induced Stat3-specific tyrosine 705 phosphorylation, while expression of dominant negative c-Src had the opposite effect (Fig. 7). c-Src kinase activity similarly affected the nuclear protein binding affinity of the Stat3 consensus sites on the *HGF* promoter (Fig. 8). We used electrophoretic mobility shift assays to examine the Stat3 consensus DNA binding affinity of nuclear protein extracts from cells expressing different mutants of c-Src. Radiolabeled oligonucleotide probes with DNA sequences corresponding to the two Stat3 consensus binding sites in the region between -254 to -70 of the *HGF* promoter were used to detect putative Stat3 binding (Fig. 8). Binding of probes corresponding to each Stat3 consensus site (-110 or -149) was detected in nuclear protein extracts of SP1 cells (lane 1 in Fig. 8, A and B, respectively). These DNA binding activities were specific since the presence of the corresponding unlabeled probes abolished the binding (second lane), while a probe with an unrelated DNA sequence had no effect (third lane). In addition, when comparing first, fourth, and seventh lanes (Fig. 8), it is apparent that there was less specific DNA binding in nuclear extracts from SP1 cells expressing dominant negative c-Src than in control cells expressing no exogenous c-Src. Moreover, nuclear extracts from SP1 cells expressing activated c-Src had higher binding activity than that from untransfected cells. This finding indicates that the expression of dominant negative c-Src reduces nuclear protein binding to the Stat3 consensus sites, whereas activated c-Src has the opposite effect. Thus specific binding of nuclear protein to the Stat3 consensus sites correlates with phosphorylation at tyrosine 705 of Stat3 in these cells.

Although there is a strong indication of Stat3 being the transcription factor binding to the c-Src responsive region of the *HGF* promoter, other Stat proteins (such as Stat1, Stat5A, and Stat5B) can also bind to a Stat3 consensus site, albeit at lower levels (28, 45, 46). Therefore, antibodies against specific Stat proteins were used in supershift experiments to determine the composition of the DNA binding complex (Fig. 9). Nuclear extracts from SP1 cells were preincubated with antibodies against Stat1, Stat3, Stat5A, or Stat5B prior to the addition of the radiolabeled probe. Both -110 (Fig. 9A) and -149 (Fig. 9B) probes formed DNA-protein complexes when nuclear extracts were added. However, only anti-Stat3 antibody could efficiently bind to these complexes to form a supershift band. Antibodies to Stat1 (data not shown), Stat5A, or Stat5B did not retard the DNA-protein complex further, despite the fact that these tran-

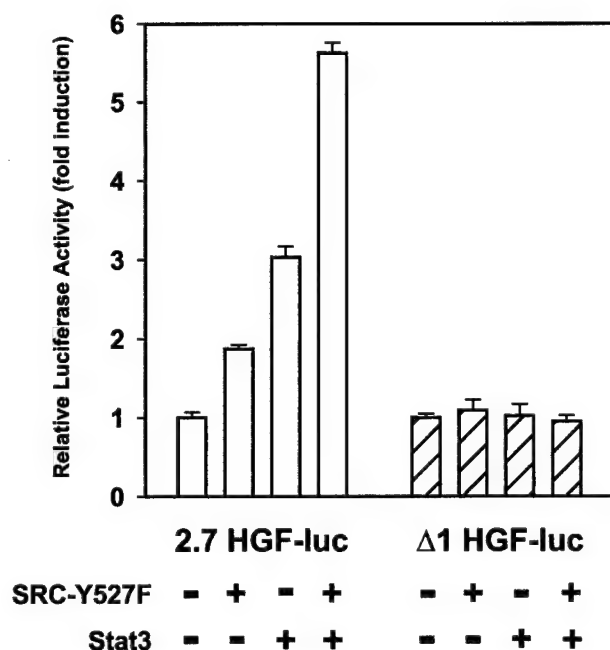


FIG. 6. The cooperative effect of Stat3 and activated c-Src on induction of HGF transcription requires the c-Src responsive region of the HGF promoter. SP1 cells were co-transfected with the 2.7 HGF-luciferase reporter, an internal deletion mutant ($\Delta 1$ HGF-luc), and a combination of activated Src (SRC-Y527F) and Stat3 as indicated. Transfections and luciferase assays were performed as described in the legend to Fig. 1B. Values represent the mean \pm S.D. of triplicate samples. The experiment was done four times with similar results.

scription factors were present in SP1 cells (data not shown). This observation suggests that Stat3 is preferentially involved in the DNA-protein complexes which bind to the c-Src responsive elements in the HGF promoter.

DISCUSSION

During normal breast development, HGF is expressed primarily by mesenchymal cells, while its receptor Met is expressed by epithelial cells (7). However, HGF is expressed in regions of human invasive breast carcinoma, and in various breast carcinoma cell lines (11–13). During tumorigenesis HGF stimulates angiogenesis, invasion, and metastasis (47, 48). Our laboratory (1) and others (49) have shown that HGF can stimulate survival of carcinoma cells. Therefore, acquired HGF expression leading to an HGF autocrine loop in breast carcinoma cells may be an important step during mammary tumorigenesis. However, the regulation of HGF expression in breast carcinoma cells is not very well understood, although some studies have been done in fibroblasts (50–54). In the present study, we examined the role of c-Src kinase, which shows increased activity in human breast cancer (55), in controlling HGF expression in breast carcinoma cells.

We previously described a mammary breast carcinoma cell line, SP1, which expresses both HGF and activated Met (23). In SP1 cells, several downstream signaling molecules, such as phosphatidylinositol 3-kinase, phospholipase C γ , and focal adhesion kinase, are constitutively phosphorylated on tyrosine residues in SP1 cells, consistent with the presence of an autocrine loop (1, 23). We have also found that c-Src tyrosine kinase in SP1 cells is constitutively active and is required for several HGF-dependent processes, such as cell motility and anchorage-independent growth (19).

In this report, we showed that inhibition of c-Src kinase activity in SP1 cells, through either the presence of c-Src kinase inhibitors or the expression of a dominant negative mutant of

c-Src, caused a decrease in HGF mRNA and protein levels. Expression of an activated c-Src kinase had the reverse effect. This finding suggests that c-Src is important in regulating the basal level of HGF transcription in epithelial and carcinoma cells, and can induce elevated expression of HGF. However, since inhibition of c-Src kinase activity cannot completely eliminate HGF basal expression, other transcription factors may play roles in maintaining HGF basal expression. Indeed, in our system, the Sp1 transcription factor is essential in maintaining HGF basal level transcription, but has no effect on c-Src-induced HGF expression (data not shown). Furthermore, aggregates of SP1 cells expressing the activated form of c-Src, in which HGF protein level was high, showed spontaneous scattering when plated on plastic, compared with the parent cell line which required addition of exogenous HGF.³ The higher level of endogenous HGF expression in SP1 cells expressing the activated form of c-Src may be sufficient to induce spontaneous scattering of these cells. Together, these findings suggest that c-Src kinase activity is important in regulating HGF expression.

By using deletion mutants of the HGF promoter, we mapped the c-Src responsive element to -254 to -70 bp. Since there is significant homology among the mouse, rat, and human HGF promoter sequences between -500 and +1 (41), the regulation of HGF expression by c-Src kinase through this element is probably conserved among these species. Previous studies in fibroblast cells have demonstrated several transcription factors which regulate HGF expression: C/EBP (-4 bp) (50), an epithelial cell-specific repressor (-16 bp) (9), Sp1/Sp3 (-318 bp) (52), estrogen receptor (-872 bp) (51), and chicken ovalbumin upstream promoter-transcription factor (-860 bp) (51). Transgenic mouse studies showed that 0.7 kb of the HGF promoter exhibited the same expression pattern as the full-length (2.7 kb) promoter (38). Although in our system we observed that Sp1/Sp3 maintain the basal level expression of HGF in breast carcinoma cells, these sites are not responsible for c-Src induced expression of HGF (data not shown). The C/EBP site appeared to have no transcriptional activity *in vivo* (38). Binding sites for estrogen receptor and chicken ovalbumin upstream promoter-transcription factor are likely to be involved in estrogen-induced expression of HGF since the upstream sequence between -2.7 and -0.7 kb has been shown to be necessary for maximal inducibility of the HGF promoter (such as after partial hepatectomy) (38). However, the c-Src responsive region (-254 to -70 bp) described here has not been previously reported.

In the c-Src responsive region of the HGF promoter there are two consensus binding sites for Stat3 (at -110 and -149), both of which are completely conserved among human, mouse, and rat. Our results showed that Stat3, in cooperation with c-Src kinase, can activate HGF promoter, this activation is completely dependent on the presence of these Stat3-binding sites and implies a role of Stat3 as a downstream effector of c-Src kinase. We therefore examined the mechanism by which c-Src regulates Stat3 activity in SP1 carcinoma cells. Stat3 has been shown to be regulated by both tyrosine and serine phosphorylations (56–58). Although there is no direct evidence that Stat3 is phosphorylated directly by c-Src, some reports suggest that c-Src and Stat3 interact physically (30, 59). Therefore, it is possible that c-Src regulates Stat3 through tyrosine phosphorylation. Our results showed that expression of a dominant negative form of c-Src reduced tyrosine phosphorylation of Stat3 and the expression of constitutively active c-Src mutant had the opposite effect. In addition, we found that the formation of a DNA-protein complex with the two Stat3-binding sites in the c-Src responsive elements was dependent on

³ B. Elliott, unpublished results.

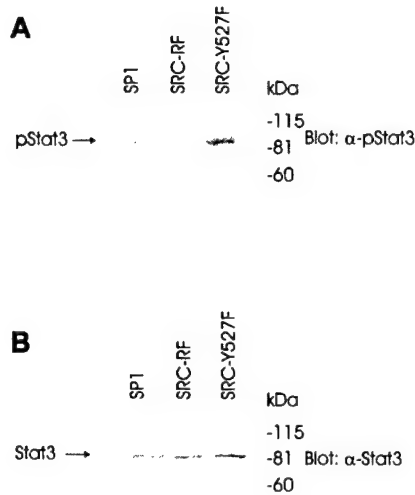


FIG. 7. c-Src kinase activity regulates phosphorylation of tyrosine residue 705 of Stat3. SP1 cells transfected with SRC-RF or SRC Y527F, or untransfected SP1 cells, were lysed. Equal amounts of proteins from each cell lysate were subjected to denaturing SDS-PAGE. The proteins were then transferred onto nitrocellulose and the blot was probed with antibody specific for phosphotyrosine 705 of Stat3 (Panel A). The blot was subsequently reprobed with anti-Stat3 (pan) antibody (Panel B).

the level of c-Src kinase activity in the cells. An apparently greater effect of activated c-Src on the binding activity of the -149 Stat3 site compared with the -110 Stat3 site was observed. This difference could potentially represent different binding affinities, or interaction with other transcription factors.

Stat2, -4, and -6 are not normally expressed in mammary tissues (60–63), and are therefore unlikely to be involved in the formation of DNA-protein complexes in SP1 cells. Both Stat1 and

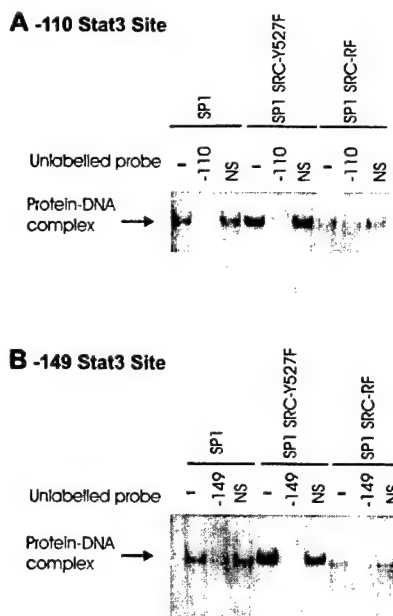


FIG. 8. c-Src kinase activity regulates nuclear protein binding to the Stat3 consensus sites (at positions -110 and -149) of the HGF promoter. Nuclear extracts were prepared from SP1 cells transfected with SRC-RF, SRC-Y527F, or untransfected cells. Equal amounts of each nuclear extract were used in binding studies with radiolabeled probes containing either the -110 (Panel A) or the -149 region (Panel B) of the HGF promoter. 10-fold molar excess of an unlabeled probe containing the -110, -149 or a nonspecific sequence (NS), respectively, was included in the binding reaction where indicated. The gel was fixed, dried, and analyzed using a Storm PhosphorImager as described under "Experimental Procedures." The arrow indicates the position of the protein-DNA complex.

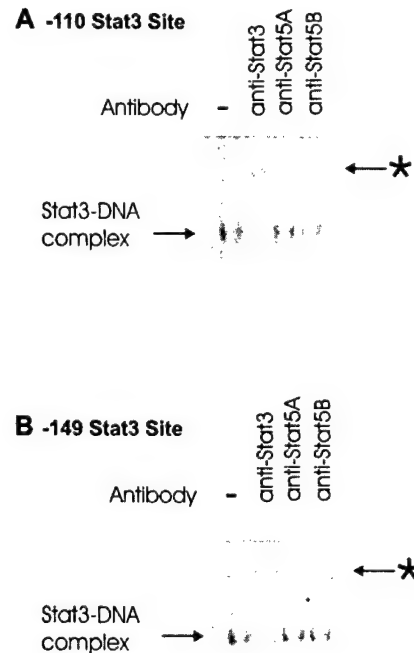


FIG. 9. Stat3 forms part of the DNA-protein complex at both the -110 and -149 consensus sites. Nuclear extracts were prepared from SP1 cells as described under "Experimental Procedures." For supershift assays, nuclear extracts were incubated with anti-Stat3, Stat5A, or Stat5B antibody on ice for 30 min prior to electrophoretic mobility shift assay analysis. After incubation with labeled -110 (Panel A) or -149 (Panel B) probes, the reaction was subjected to nondenaturing PAGE. The asterisk indicates the position of supershift band.

Stat3 have been shown to be activated by c-Src in fibroblast cells when they are stimulated with various growth factors (30, 45, 64), while Stat5 is expressed and activated during mammary development (60). Moreover, both Stat3 and Stat5 have been found to be constitutively active in cells transformed by v-Src, v-Abl, and other oncoproteins (42–44, 59, 65–67). Therefore, other Stat proteins cannot be ignored as part of the complex. Supershift studies with antibodies against specific Stat proteins allowed us to identify Stat3, and exclude Stat1, -5A, or -5B, as a component of the DNA-protein complex. Furthermore, since there is only one DNA complex formed with each probe and each probe can effectively abolish DNA-protein complex formation with the other (data not shown), the same DNA-binding protein(s) must be involved in binding to each of these regions. Since Stat3 protein binds as dimers to its binding sites, it is reasonable to assume that Stat3 dimers are binding to both sites in the c-Src responsive region. Together, these observations suggest that c-Src kinase may regulate Stat3-dependent transcriptional activation through direct or indirect tyrosine phosphorylation of Stat3, resulting in increased DNA binding ability.

In contrast to SP1 carcinoma cells, the nonmalignant mammary epithelial cell line, HC11, showed a very low level of HGF transcription with no detectable HGF protein. Furthermore, expression of activated c-Src (Y527F) had very little effect on HGF transcription in HC11 cells, possibly due to the presence of the epithelial cell type-specific repressor (9). However, coexpression of Stat3 and activated c-Src caused a strong synergistic induction of HGF transcription in HC11 cells, implying that the lack of c-Src kinase activity and the low level of activated Stat3 may be limiting for HGF transcription in HC11 cells. Increased activities of these proteins can possibly override the repression by the cell type-specific repressor and allow expression of HGF in epithelial cells. Interestingly, we found that fibroblast cells, which normally express HGF, also require c-Src kinase activity to regulate HGF expression, and that this

regulation of HGF is dependent on the same region of the *HGF* promoter as our breast carcinoma cell model (data not shown). These results suggest a similar regulation pattern between fibroblast cells that express HGF endogenously, and carcinoma cells, which acquire the ability to express HGF. It is possible that during epithelial-mesenchymal transition, epithelial cells acquire different genetic mutations leading to the activation of c-Src kinase. For example, increased expression and activity of HER2/Neu, an epidermal growth factor-like receptor tyrosine kinase, in breast carcinoma cells has been shown to activate c-Src kinase (64, 68). Activation of c-Src, in turn, may lead to a de-repression of HGF expression, giving these cells a growth advantage compared with nontransformed epithelial cells. This step may be an important initial step in tumorigenesis.

Here, we have reported that c-Src kinase and Stat3 act cooperatively in stimulating *HGF* gene expression in breast carcinoma cells, most likely via regulation of Stat3-dependent transcriptional activation of the *HGF* promoter. Although many reports have indicated that increased Src kinase activity (particularly through the expression of v-Src) can activate gene expression via Stat3, in this study we identify a target region (−254 to −70 bp) of the *HGF* promoter responsive to elevated activity of c-Src kinase in breast carcinoma cells. There is recent evidence suggesting that an HGF autocrine loop can provide selective survival and growth advantage to carcinoma cells and that overexpression of HGF can be a reliable indicator of poor survival of breast cancer patients (11). Our findings therefore provide an important link between breast cancer progression and HGF expression, and suggest that the c-Src/Stat3 pathway regulating HGF expression can be a potential target for therapy in breast cancer treatment.

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The role of hepatocyte growth factor (scatter factor) in epithelial-mesenchymal transition and breast cancer¹

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Abstract: North American women have a one in eight lifetime risk of developing breast cancer, and approximately one in three women with breast cancer will die of metastases. We, and others, have recently shown that high levels of expression of hepatocyte growth factor (HGF) and its receptor Met are associated with invasive human breast cancer and may be causally linked to metastasis. This high level of HGF and Met expression has been considered as a possible indicator of earlier recurrence and shortened survival in breast cancer patients. In contrast, HGF expression (but not Met) is strongly suppressed in normal breast epithelial cells. HGF and Met are therefore candidate targets for therapeutic intervention in the treatment of breast cancer. We have recently demonstrated that sustained activation or hyper-activation of c-Src and Stat3, which occurs in invasive breast cancer, can stimulate strong expression of HGF in carcinoma cells. In contrast, transient induction of Stat3 occurs in normal epithelium and promotes mammary tubulogenesis. We hypothesize that increased autocrine HGF-Met signaling is a critical downstream function of c-Src-Stat3 activation in mammary tumorigenesis. Future studies will identify novel Stat3 consensus sites that regulate HGF promoter activity and HGF expression preferentially in carcinoma cells and could lead to novel therapeutic drugs that specifically block HGF expression in mammary carcinoma cells, and which could be used in combined treatments to abrogate metastasis.

Key words: HGF, Src-Stat3 signaling, epithelial-mesenchymal transition, breast cancer.

Résumé : En Amérique du Nord, une femme sur huit risque de souffrir d'un cancer du sein au cours de son existence, et environ une femme sur trois mourra des métastases. À l'instar d'autres chercheurs, nous avons montré récemment que de hauts taux d'expression du facteur de croissance des hépatocytes (HGF), et de son récepteur Met, sont associés au cancer du sein envahissant chez l'humain et pourraient être directement liés à la métastase. Ce haut taux d'expression du HGF et du Met a été considéré comme un indicateur possible de récurrence précoce et d'abrègement de la survie chez les patientes atteintes d'un cancer du sein. En revanche, l'expression du HGF (mais pas du Met) est fortement réduite dans les cellules épithéliales du sein normal. Le HGF et le Met sont, par conséquent, des cibles d'intervention thérapeutique dans le traitement du cancer du sein. Nous avons démontré récemment que l'activation soutenue ou l'hyperactivation de c-Src et de Stat3, qui se produit dans le cancer du sein envahissant, pourrait stimuler la forte expression du HGF dans les cellules de carcinomes. Par contre, l'induction transitoire de Stat3 se produit dans l'épithélium normal et favorise la tubulogenèse mammaire. Nous émettons l'hypothèse qu'une signalisation autocrine accrue du complexe HGF-Met est une importante fonction en aval de l'activation du complexe c-Src-Stat3 dans la tumorigenèse mammaire. De futures études identifieront de nouveaux sites consensuels de Stat3, régulant l'activité promotrice du HGF et l'expression du HGF de préférence dans les cellules de carcinomes, qui pourraient mener à la mise au point de médicaments thérapeutiques bloquant spécifiquement l'expression du HGF dans les cellules de carcinomes mammaires et pouvant être utilisés dans des traitements combinés pour supprimer la métastase.

Mots clés : HGF, Src-Stat3, transition épithéliale - mésenchymateuse, cancer du sein.

[Traduit par la Rédaction]

Introduction to mammary gland development

In normal mammary gland development, branching tubulogenesis develops from a single mammary bud that un-

dergoes elongation and side-branching at puberty to infiltrate the entire mammary fat pad (Medina and Daniel 1996; Richert et al. 2000; Ronnov-Jessen et al. 1996). Stromal interactions are critical in mammary tubulogenesis (Wood-

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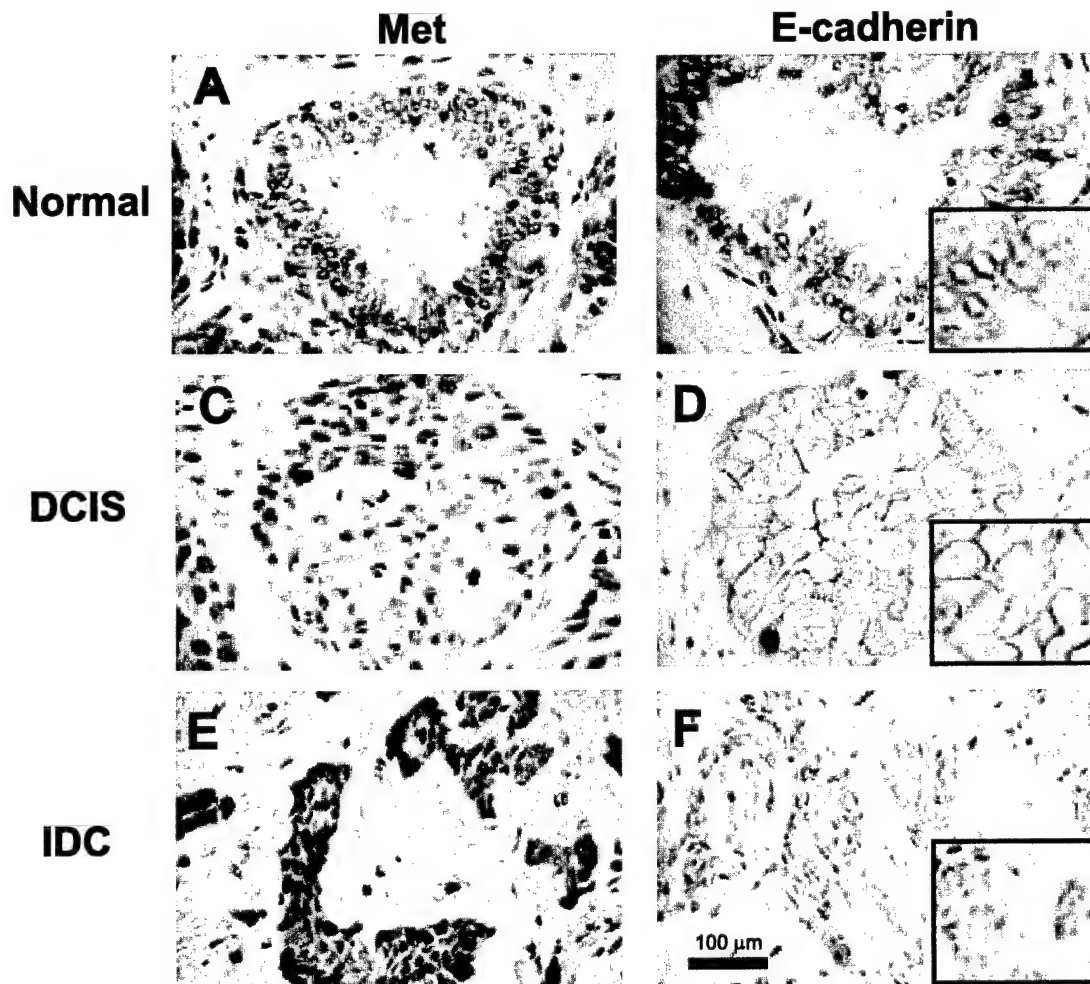
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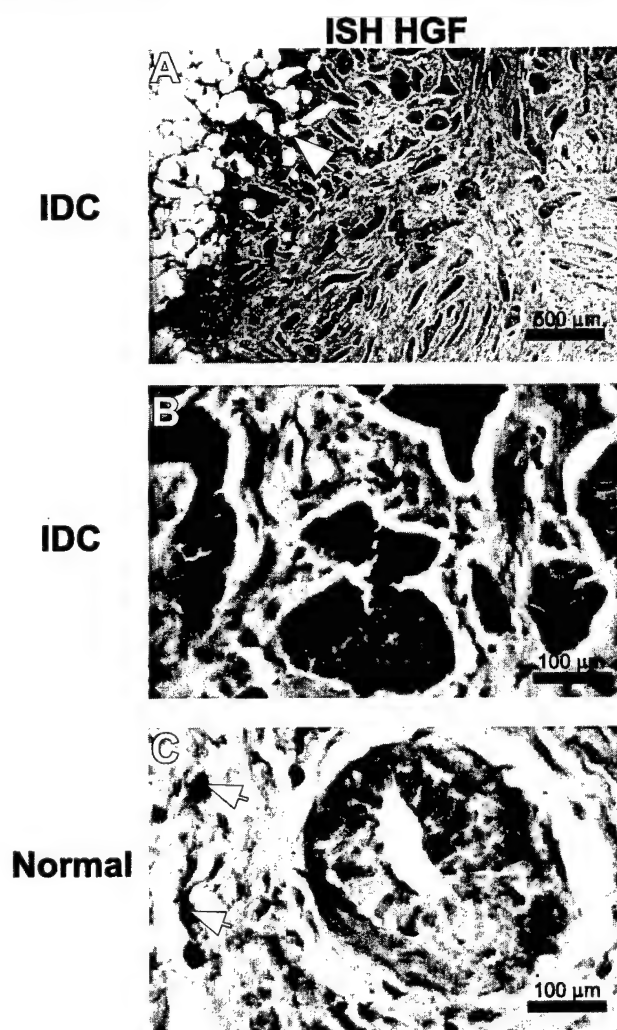
Fig. 1. Immunoperoxidase localization of Met and E-cadherin in normal ducts (A and B), ductal carcinoma in situ (DCIS) (C and D), and invasive ductal carcinoma (IDC) (E and F) from human breast cancer tissues. (A, C, E) Staining with anti-Met antibody. (B, D, F) Staining with anti-E-cadherin antibody. Staining for Met was relatively consistent in all epithelial regions (normal and malignant), but was more intense in IDC than in benign ducts or DCIS. In contrast, E-cadherin staining was strong with accentuation at cell-cell contacts in normal epithelium and DCIS, whereas E-cadherin staining was frequently reduced or absent in IDC. The scale bar refers to the lower magnification in each panel. A 4-fold higher magnification is shown in the insets.



ward et al. 1998). The estrogen signal, a key initiator of this process, is thought to be delivered at least in part via the mammary stroma (Cunha and Hom 1996), which secretes growth factors (e.g., hepatocyte growth factor (HGF; Soriano et al. 1998; Rahimi et al. 1994), also known as scatter factor, and keratinocyte-derived growth factor (Bansal et al. 1997)) that act in a paracrine manner on adjacent basal (progenitor) epithelial cells (Smith and Chepko 2001). The basal cells at the stromal-epithelial interface secrete transforming growth factor α (TGF- α) (Snedeker et al. 1991), which promotes autocrine activation of epidermal growth factor (EGF) receptor and basal cell proliferation. Metalloproteinases (e.g., MMP3; Rudolph-Owen and Matrisian 1998) are also required as the dividing basal cells migrate through the interstitial tissue of the fat pad. As the basal cells divide, they move away from the stromal inter-

face and align to form a polarized luminal epithelium (keratins 8/18 positive) with clearly defined cadherin-based adherens junctions and desmosome-based tight junctions. Myoepithelial cells (keratins 5/14 positive), which are thought to be derived from basal cells (Petersen et al. 2001) and display smooth muscle-like characteristics, form an outer layer surrounding the luminal cells. TGF- β , produced in epithelial cells and adjacent stroma, promotes a differentiated epithelial phenotype and stimulates deposition of extracellular matrix (ECM) proteins and formation of a basement membrane sheath around the entire duct (Daniel et al. 1996). The regulation of mammary gland development is a complex cascade of intercellular interactions beyond the scope of this review, and has been extensively reviewed elsewhere (Robinson et al. 1999; Werb et al. 1996; Dunbar and Wysolmerski 2001).

Fig. 2. In situ hybridization (ISH) analysis of HGF mRNA expression in invasive ductal carcinoma (IDC) and normal ducts from human breast cancer tissues. ISH analysis of HGF mRNA expression in IDC (A, B) and normal ducts (C) from human breast cancer tissues was carried out as described previously (Tuck et al. 1996). Strong positivity for HGF mRNA was observed at the advancing front of invasive breast carcinomas (A, arrowhead; B). In contrast, central regions of invasive tumors frequently showed less intense expression of HGF mRNA (right hand side of panel A). Normal ducts showed weak expression of HGF mRNA, although variable sometimes intense staining was seen in the peri-epithelial stromal cells (C, arrowheads). Scale bars are indicated.



Epithelial-mesenchymal transition in breast cancer

North American women have a one in eight risk of developing breast cancer in their lifetime (Lippman et al. 2001). Development of a malignant phenotype is a multi-step process, characterized by the loss of epithelial polarity, disper-

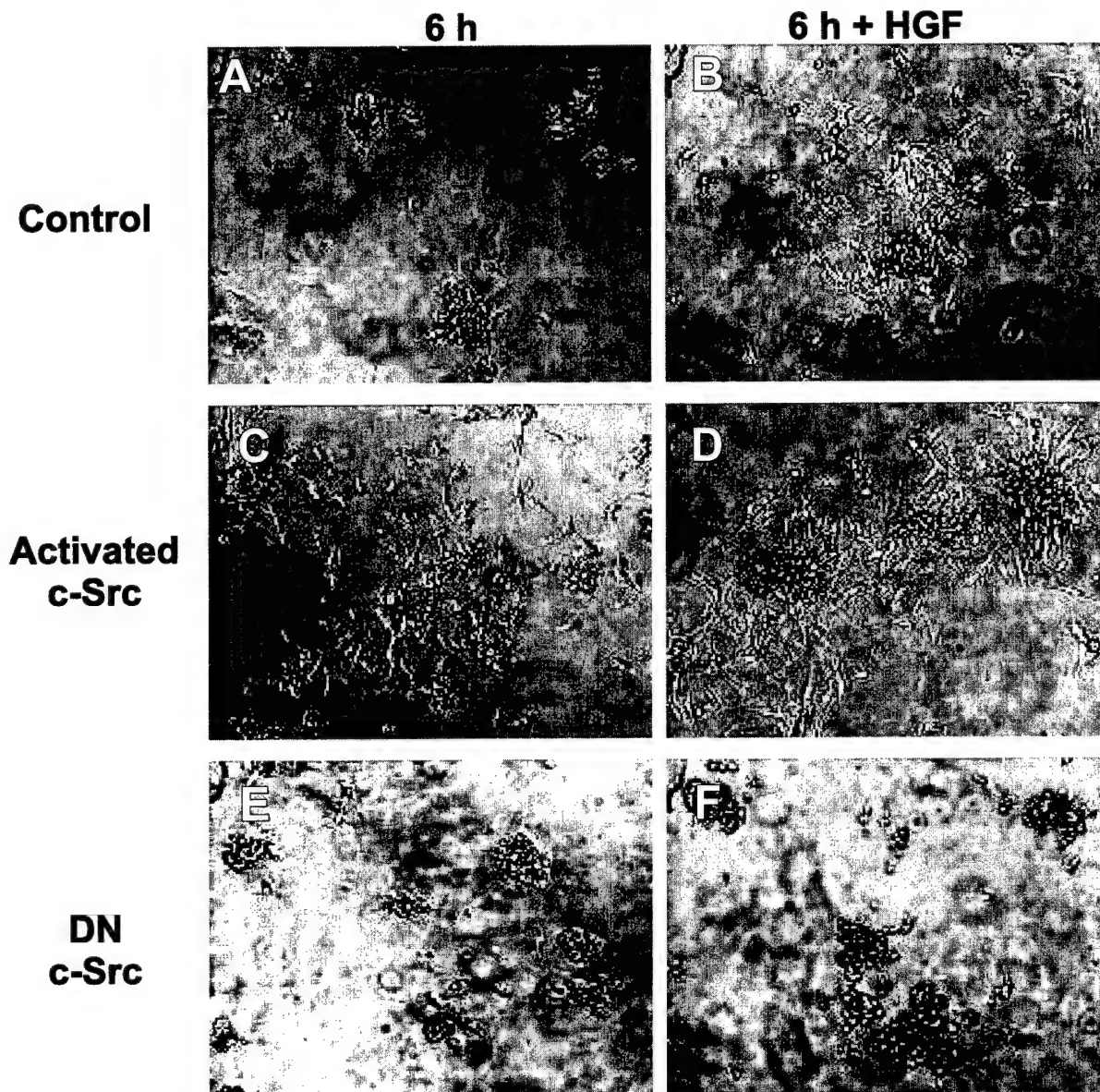
sion of cell-cell junctions, degradation of basement membrane, and increased cell migration and invasion (Boyer et al. 1996; Birchmeier et al. 1996). This process, referred to as epithelial-mesenchymal transition (EMT), is indicative of invasion by breast carcinoma cells, earlier recurrence, and shortened patient survival. EMT is caused by multiple genetic changes, including activating mutations in receptor tyrosine kinases (RTKs; e.g., HER2/neu (Chan et al. 1999)) and loss of function of adhesion molecules such as E-cadherin, β -catenin (Birchmeier et al. 1995), and adenomatous polyposis coli (APC) protein (Barth et al. 1997).

HGF and Met regulate both morphogenic and tumorigenic phenotypes

HGF is a potent inducer of EMT in many epithelial systems (Birchmeier et al. 1996), although other growth factors (e.g., FGF and EGF) may also be involved (Boyer et al. 1996). HGF is a multi-functional cytokine that stimulates morphogenesis, cell survival (Bowers et al. 2000; Qiao et al. 2000), mitogenesis, motility, invasion, and metastasis (Lamszus et al. 1997). In normal mammary development, HGF is produced by mesenchymal cells and, along with other growth factors (e.g., neuregulin; Yang et al. 1995) and the stromal protein epimorphin (Hirai et al. 1998), stimulates tubulogenesis in a tightly controlled paracrine manner (Brinkmann et al. 1995). However, over-expression of HGF and Met occurs in many types of invasive cancers, including breast carcinomas (reviewed in Tuck et al. 1996). Furthermore, diverse tumorigenesis in a broad range of tissues occurs in transgenic mice over-expressing HGF or constitutively activated Tpr-Met (Otsuka et al. 2000; Takayama et al. 1997; Liang et al. 1996). Ectopic over-expression of HGF and Met also causes transformation in a variety of epithelial cell lines (Fixman et al. 1995; Bellusci et al. 1994). Thus, a shift from transient activation of Met to sustained high levels of Met activation (Marshall 1995) and co-operativity with other RTKs (e.g., HER2/neu (Andrechek and Muller 2000)) can cause a switch in the HGF response from morphogenesis to tumorigenesis.

Recent studies have identified high levels of HGF and Met expression in breast carcinoma cells as a possible independent predictor of recurrence and shortened survival in breast cancer patients (Yamashita et al. 1994; Jin et al. 1997; Ghossein et al. 1998; Toi et al. 1998). Sustained activation of HGF-Met signaling is associated with dissociation of cadherin-based adherens junctions, followed by loss of cadherin expression (Zschiesche et al. 1997). The modulation of adherens junctions by HGF-Met involves phosphorylation of β -catenin, leading to its reduced affinity for the E-cadherin complex and subsequent degradation (Hiscox and Jiang 1999; Huber et al. 1996). In normal breast epithelium and ductal carcinoma in situ (DCIS), strong expression of E-cadherin with accentuation at cell-cell contacts is evident (Figs. 1B and 1D). In contrast, decreased expression of E-cadherin frequently occurs in invasive ductal carcinoma (IDC) (Fig. 1F), while Met expression is relatively consistent throughout, and most intense in IDC (Figs. 1A, 1C, and 1E). Although HGF and Met mRNA are differentially expressed in normal stromal and epithelial

Fig. 3. c-Src kinase activity is required for HGF-induced dispersion of breast carcinoma cells. SP1 breast carcinoma cells were transfected with activated c-Src (C, D), dominant negative (DN) c-Src (E, F), or vector alone (control; A, B). Each cell line was allowed to form aggregates in suspension rotating cultures overnight in RPMI with 0.5 mg/mL BSA. Cell aggregates were washed and plated on plastic with (B, D, F) or without (A, C, E) HGF (30 ng/mL) for 6 h. The representative photographs show that activated c-Src induced spontaneous dispersion of aggregates compared with control cells, which required stimulation with HGF for dispersion. In contrast, DN c-Src inhibited aggregate dispersion even in the presence of HGF.



cells, respectively, (Brinkmann et al. 1995), it was not clear what cell types produce HGF and Met in breast carcinomas. Using in situ hybridization (ISH), we have shown that invasive human breast carcinoma cells frequently show strong HGF mRNA expression, particularly at the migrating tumor front, compared with weak expression in more central regions of invasive tumor (Figs. 2A and 2B). Met mRNA showed a similar gradient of strong expression at the stromal interface to weak expression in the central regions of the tumor (Tuck et al. 1996). In contrast, HGF mRNA is weakly

expressed in normal epithelium (Fig. 2C), although some epithelial expression of HGF mRNA in certain instances (such as hyperplasia) was evident (Tuck et al. 1996; Wang et al. 1994). Jin et al. (1997) have reported a similar gradient of HGF and Met expression from normal breast – benign hyperplasias (lowest), to DCIS (higher), to invasive carcinoma (highest). Together, these findings raise the possibility that a high level of HGF expression and sustained activation of Met (referred to as an HGF–Met autocrine loop) in breast carcinoma cells can promote increased EMT, invasion, and

metastasis. Support for this contention was provided by Vande Woude (Cao et al. 2001), who showed that an anti-HGF neutralizing antibody combination can inhibit growth of human glioblastoma xenografts that are dependent on an HGF-Met autocrine loop. Further studies are needed to test the effect of HGF neutralizing antibodies on growth and metastasis of breast carcinoma cells.

c-Src kinase is a key regulator of EMT in breast carcinoma cells

The non-RTK c-Src is expressed in many cell types and is required for normal mammary ductal development (W.J. Muller, personal communication). Hyper-activation of c-Src occurs in many human cancers (Ottenhoff-Kalff et al. 1992) by a variety of mechanisms including increased RTK (e.g., HER2/neu (Muthuswamy and Muller 1995)) or integrin signaling (Bjorge et al. 2000), dephosphorylation of a negative regulatory tyrosine Y529 (Bjorge et al. 2000), or mutation (Irby et al. 1999). c-Src plays a critical role in breast cancer; however, activation of c-Src alone is not sufficient for mammary tumorigenesis in transgenic mice (Guy et al. 1994), indicating that c-Src must interact with other signaling pathways. c-Src is recruited to focal adhesions and cell-cell contacts (McLean et al. 2000) and is known to modulate cell proliferation, spreading, and migration (reviewed in Rahimi et al. 1998). Activation of c-Src causes increased turnover of focal adhesions on ECM substratum and is required for scattering of carcinoma cells (Fig. 3; Owens et al. 2000). Activated c-Src can also induce expression of many genes, including cellular growth factors such as vascular endothelial growth factor (Mukhopadhyay et al. 1995), WAF1/CIP1, and cyclin D1 (Sinibaldi et al. 2000).

c-Src kinase is required for HGF expression in breast carcinoma cells

Since c-Src is activated in most human and mouse breast carcinomas, we examined the role of c-Src in HGF expression in a mouse breast carcinoma cell line SP1, which co-expresses HGF and activated Met. We showed that HGF mRNA and protein levels increased in tumor cells expressing a constitutively activated c-Src mutant, while a dominant negative (DN) c-Src mutant or an inhibitor of c-Src family kinases (PP2) (Hung and Elliott 2001) had the opposite effect (Fig. 4A). These data suggest that HGF expression (at both mRNA and protein levels) is regulated by c-Src kinase activity. To determine the effect of c-Src kinase mutants on *HGF* promoter activity, we constructed a reporter plasmid (HGF-luc) with the luciferase gene linked to the 2.7 kb region 5' of the *HGF* transcriptional start site (Fig. 4B). The 2.7 kb 5' upstream segment of the promoter has been shown previously to be sufficient to support *HGF* transcriptional activity in transgenic mice (Bell et al. 1998). Co-transfection of the HGF-luc construct with an activated c-Src mutant increased *HGF* promoter activity, whereas a DN c-Src mutant had the opposite effect. Thus *HGF* transcription is strongly responsive to c-Src. Using a deletion analysis of the *HGF* promoter, we identified a novel region between -254 and -70 bp, which is required for c-Src responsiveness of *HGF*

Fig. 4. c-Src modulates HGF mRNA expression and *HGF* promoter activity. (A) SP1 cells transfected with dominant negative (DN) c-Src, activated c-Src, or vector alone (control) (see Fig. 3) were serum starved overnight. A nonmalignant mammary epithelial cell line, HC11, was used as a negative control. Total RNA was isolated, and the amount of HGF mRNA in each sample was determined using RT-PCR, normalized to β -glucuronidase mRNA, and expressed as the percentage of control. Expression of DN c-Src reduced the level of HGF mRNA, whereas expression of activated c-Src increased the level of HGF mRNA by 2.5-fold. (B) A reporter construct consisting of the 2.7 kb 5' *HGF* promoter region upstream of the transcriptional start site ligated to the luciferase gene (2.7 HGF-luc) or vector alone was co-transfected transiently into SP1 cells with DN c-Src or activated c-Src or vector alone. Luciferase activity of each sample was determined and normalized to the empty vector control value within each group, as described previously (Hung and Elliott 2001). Expression of the DN c-Src inhibited *HGF* promoter activity, whereas expression of activated c-Src increased *HGF* promoter activity by 2-fold (modified from Hung and Elliott 2001).

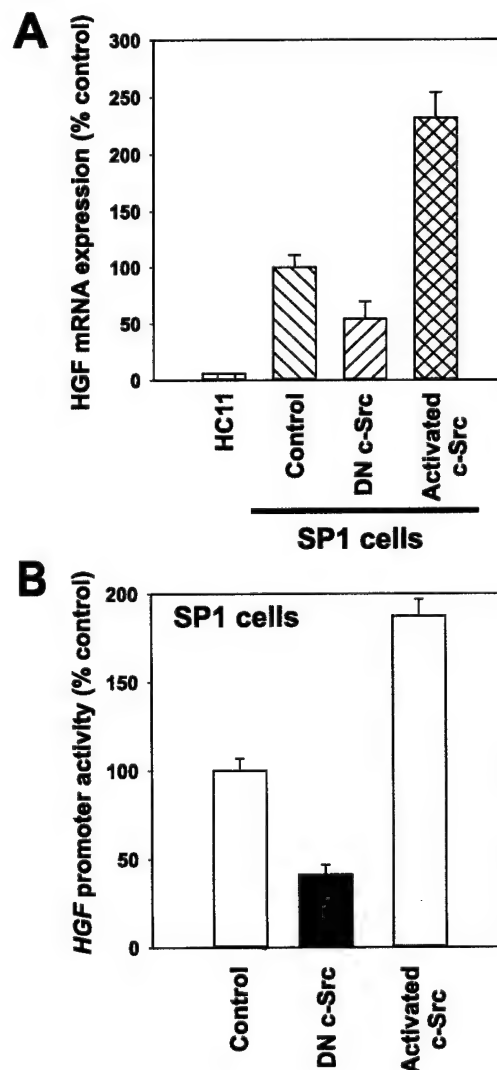
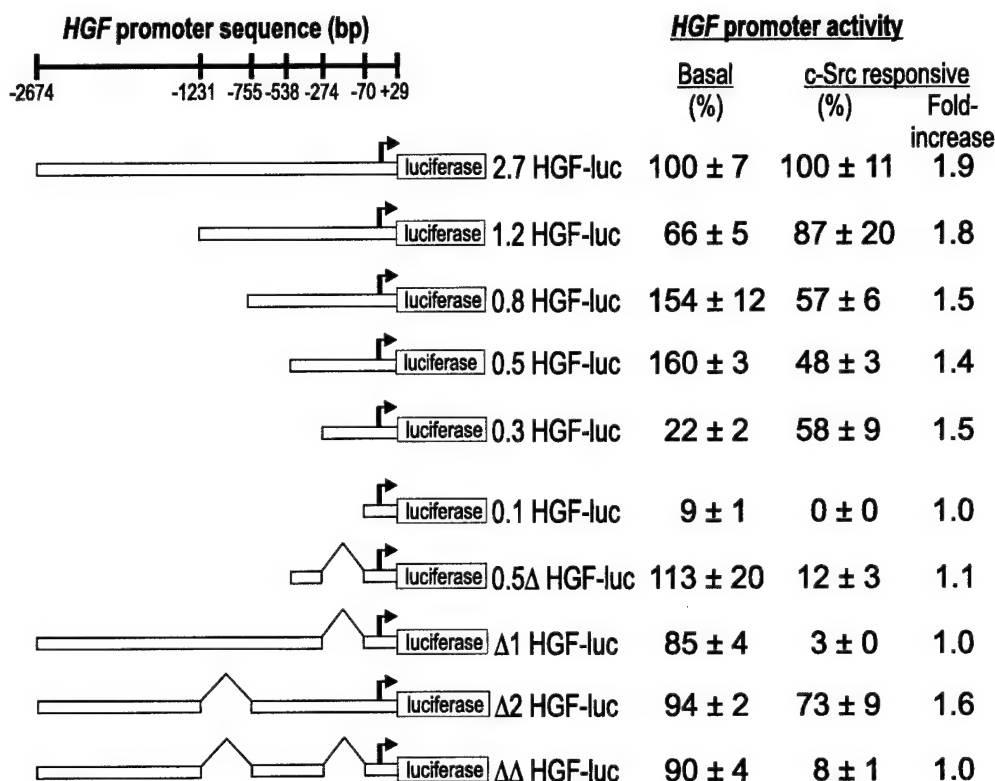


Fig. 5. Identification of the c-Src kinase responsive region (–254 to –70 bp) of the *HGF* promoter. The 2.7 HGF-luc reporter, or HGF-luc reporter constructs containing various deletions of the *HGF* promoter, were co-transfected into SP1 cells with activated c-Src, dominant negative (DN) c-Src, or empty vector. Luciferase activity of each sample was determined and normalized within each group, as described in Fig. 4. Basal activity in each group is expressed as a percentage of that of the full length promoter (2.7 HGF-luc). c-Src responsive activity of each HGF-luc promoter construct is calculated relative to the basal level in each group and is expressed as a percentage of that of the 2.7 HGF-luc reporter. The fold-increase in c-Src responsive *HGF* promoter activity is also indicated. The results show that deletions up to –538 bp (0.5 HGF-luc) had no significant effect on the c-Src responsiveness of the *HGF* promoter. A further deletion up to –273 bp (0.3 HGF-luc) significantly reduced the basal activity of the promoter, while some c-Src responsiveness remained. The remaining c-Src responsiveness was eliminated when all but 72 bp (0.1 HGF-luc) of the *HGF* promoter remained. Internal deletion constructs (0.5Δ HGF-luc and 2.7Δ HGF-luc) lacking the region between –254 and –70 bp of the *HGF* promoter did not respond to expression of activated c-Src, although basal activity remained. Detailed results are published elsewhere (Hung and Elliott 2001).



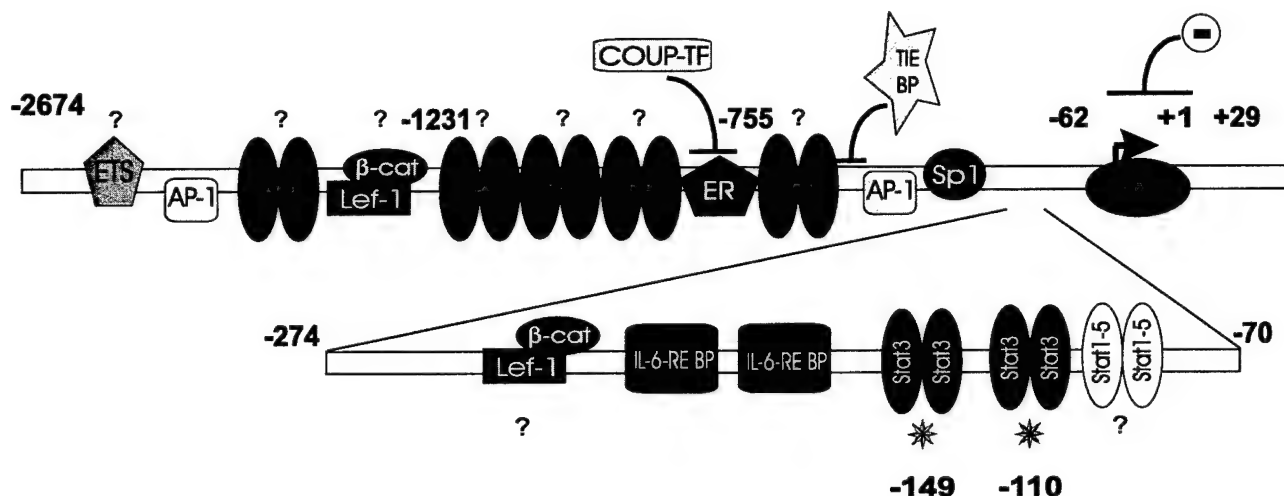
promoter activity in carcinoma cells (Figs. 5 and 6). Deletion of this region had no effect on basal activity of the full-length HGF promoter, suggesting that the c-Src responsive effect is distinct from other regulatory factors.

Organization of the *HGF* promoter

Previous studies in fibroblast cells have demonstrated several transcription factors that regulate *HGF* promoter function (Fig. 6) (reviewed in Liu et al. (1994b)). These include C/EBP (binding to a site at –9 to –4 bp) (Jiang and Zarnegar 1997), which mediates inducibility by various cytokines; Sp1/Sp3 (binding at –318 to –308 bp) (Jiang et al. 1997a), which is required for maintenance of basal transcription; and Il-6-RE binding protein (BP) (binding at –211 to –206 bp and –188 to –182 bp). In addition, estrogen receptor (ER) (binding at –872 to –860 bp) mediates estrogen inducibility of the promoter (Jiang et al. 1997b). The nuclear orphan re-

ceptor chicken ovalbumin upstream promoter-transcription factor (COUP-TF) also binds with high avidity at this site; however, ER can compete with COUP-TF binding and thereby reverse the suppressive effect of COUP-TF on *HGF* transcription. Other repressor factors include AP2 (binding at –230 to –260 bp) (Jiang et al. 2000a) and TGF-β inhibitory (TIE) BP (binding at –364 to –355 bp) (Liu et al. 1994b). Members of the upstream stimulator factor (USF) and nuclear factor-1 (NF-1) transcription factors also bind strongly to the AP2 site (Jiang et al. 2000b); NF-1 family factors suppress, while USF factors activate, *HGF* promoter function. A putative AP1 consensus site is located 13 bp downstream of the TIE-BP site, and binding of the respective factors to these adjacent sites could further modulate *HGF* transcription (Liu et al. 1994b). Thus, multiple transcription factors link *HGF* promoter activity with many growth factors, cytokines, and steroid hormones. A negative regulatory site (at –16 to +4 bp) is required for suppression

Fig. 6. Proposed organization of the *HGF* promoter. The relative positions of various putative (indicated by "?") and established consensus sites in the 2.7 kb 5' region of the *HGF* promoter upstream of the transcriptional start site are shown (see text for details). The c-Src responsive region is enlarged, and the Stat3 consensus sites at positions -149 and -110 bp are indicated by stars. The negative regulatory region (-16 to +11 bp) responsible for suppression of *HGF* transcription in epithelial cells is shown by a circled negative sign. β -cat, β -catenin.



of *HGF* transcription in normal epithelial cells and suppresses induction of *HGF* transcription by most paracrine activators (Liu et al. 1994a). The exact mechanism of this strong suppressive effect on *HGF* transcription is not known; however, it likely plays a key role in maintaining the differentiated state of mammary epithelial cells.

Interestingly, the c-Src responsive region of the *HGF* promoter contains two putative consensus sequences for the signal transducer and activator of transcription-3 factor, Stat3 (at -149 kb and -110 kb), as well as one putative Stat5 consensus site (at -96 to -87 kb) (Fig. 6; Hung and Elliott 2001). This region is distinct from the negative regulatory site involved in cell-type specific transcriptional repression of *HGF* promoter activity in epithelial cells or sites involved in cytokine- and estrogen-responsive promoter activity.

Role of Stats in normal and malignant breast development

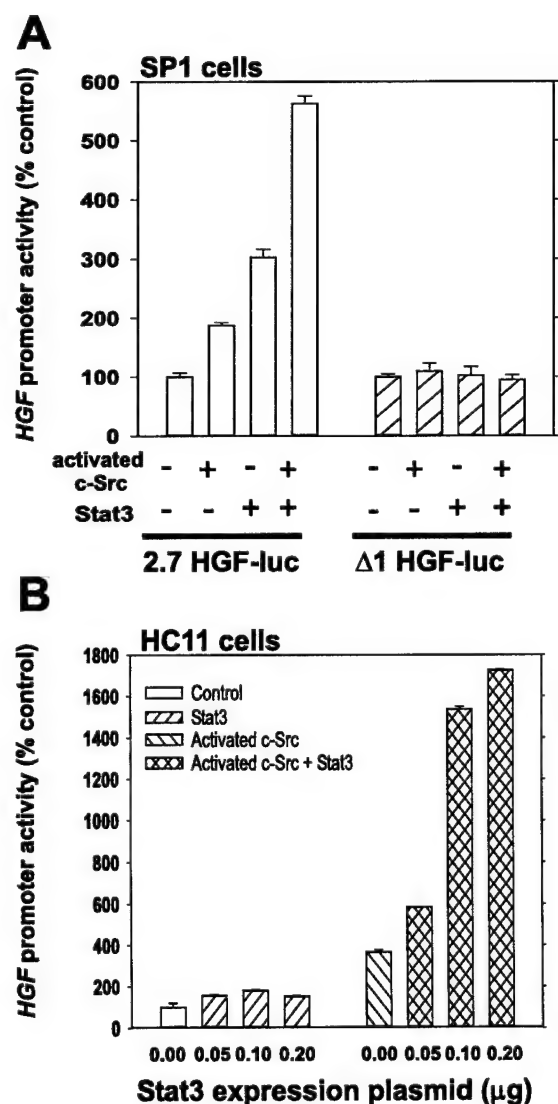
The Stat family proteins, originally identified as transcription factors mediating most cytokine signaling through Jaks, have recently been shown to be downstream of a wide variety of growth factor RTKs as well as some non-receptor tyrosine kinases (e.g., v-Src and Abl) (Bowman et al. 2000; Garcia and Jove 1998). Stat5A knockout (KO) mice are phenotypically normal; however, the mammary glands in these mice fail to develop during pregnancy or to lactate (Liu et al. 1997). In contrast, Stat3 KO mice die in utero (Takeda et al. 1997), whereas conditional KO of Stat3 in the mammary gland inhibits epithelial apoptosis and delays involution (Chapman et al. 2000; Chapman et al. 1999). The reciprocal effect of Stat5 and Stat3 on lactation and involution suggests complex roles of these Stats in the regulation of apoptosis and development in the mammary gland.

Previous reports have shown sustained high level of Stat3 activity in many human cancers including breast cancer (Bowman et al. 2000); in contrast, normal cells exhibit rapid transient induction of Stat3 activity. In addition, inhibition of Stat3 activity in human tumor cells results in cell death and (or) growth arrest preferentially in malignant cells (Catlett-Falcone et al. 1999; Niu et al. 2001b). Evidence indicates that activation of Stat3 stimulates production of soluble factors that suppress pro-apoptotic molecules (e.g., TRAIL (Niu et al. 1999a)) and induce anti-apoptotic molecules (e.g., Bcl-XL; Grandis et al. 2000) in some tissues including lymphoid and myeloid cells and in many cancers including breast carcinomas and myelomas. The mechanism that shifts the Stat3 phenotype from anti-apoptotic to apoptotic in the mammary gland is not known.

Co-operative effect of c-Src and Stat3 in stimulating *HGF* transcription and EMT

Since Stat3 activation by c-Src induces specific gene expression and is required for cell transformation, we examined whether c-Src interacts with Stat3 in the regulation of *HGF* transcription. A strong co-operative effect of c-Src and Stat3 in the activation of *HGF* transcription was observed in both breast carcinoma (SP1) and epithelial (HC11) cells (Fig. 7). The c-Src-Stat3 co-operativity in the induction of *HGF* transcription required the c-Src responsive region of the *HGF* promoter (Hung and Elliott 2001). We also showed that c-Src kinase activity increased tyrosine 705 phosphorylation and DNA binding affinity of Stat3 (but not Stat1, 5A, or 5B) to the putative consensus sites (at -110 and -149 bp). In addition, co-expression of activated c-Src and Stat3 in mammary epithelial cells showed marked cell scattering and loss of cell-cell contacts compared with cells expressing activated c-Src, or Stat3, or vector alone (B. Elliott,

Fig. 7. Co-operative effect of activated c-Src and Stat3 in activation of *HGF* transcription in breast carcinoma and epithelial cells. (A) SP1 cells were co-transfected with the 2.7 *HGF*-luc reporter, an internal deletion mutant ($\Delta 1$ *HGF*) of the 2.7 *HGF*-luc reporter construct (Fig. 5), and combinations of activated c-Src and Stat3 as indicated. After 48 h, luciferase assays were performed as described in Figs. 3 and 4. The results show a strong co-operative effect of activated c-Src and Stat3 on *HGF* transcription, which is dependent on the c-Src responsive region of the *HGF* promoter. (B) HC11 mammary epithelial cells were transfected transiently with the 2.7 *HGF*-luc reporter and activated c-Src or an empty vector (control), in combination with varying amounts of Stat3. After 48 h, luciferase assays were performed, and results are expressed as in Fig. 4. The results show very low levels of *HGF* transcription in HC11 cells expressing Stat3, activated c-Src, or empty vector alone. However, co-expression of activated c-Src and Stat3 induced *HGF* transcription up to 17-fold. Reprinted with permission from Hung and Elliott (2001).

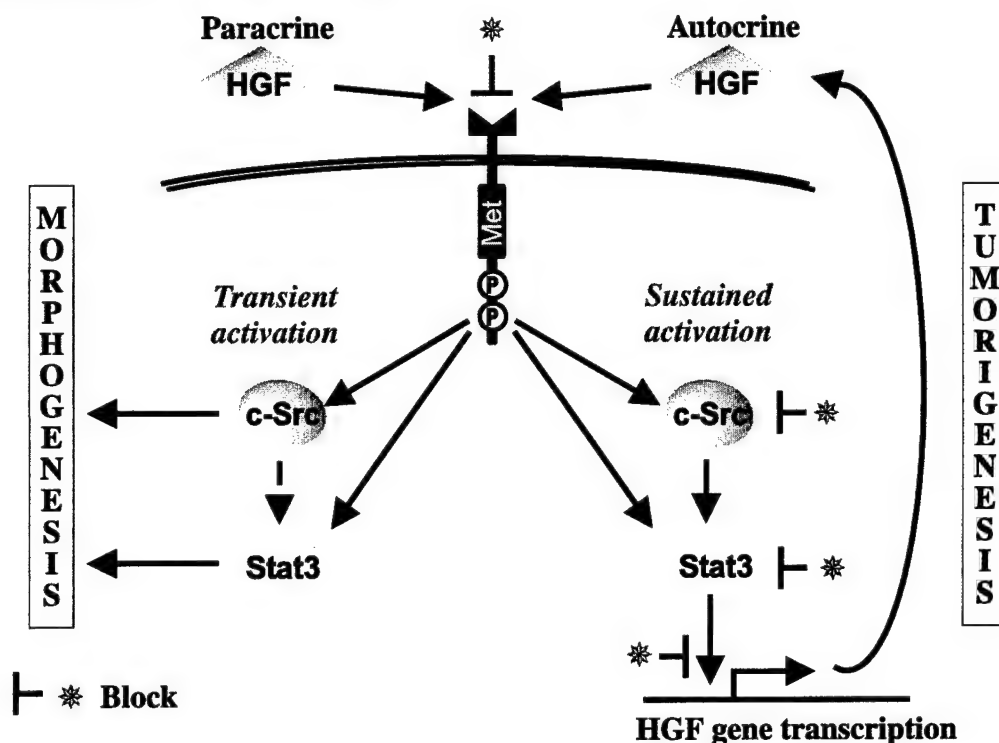


unpublished observations). Our data indicate that hyper-activation of a novel c-Src-Stat3 pathway induces *HGF* transcription and protein expression in breast carcinoma cells. This process may be important in overriding the strong repression of *HGF* transcription in normal epithelium, thereby promoting HGF protein expression, autocrine activation of Met, and mammary tumorigenesis.

HGF-Met autocrine loops as a potential target for the treatment of breast cancer metastasis

As discussed in this review, increased expression of HGF and sustained autocrine activation of Met are important steps in breast cancer invasion. The ability of tumor cells to grow and invade at distant sites is also believed to be important in metastasis. HGF and Met are therefore candidate targets for therapeutic intervention in the prevention and (or) treatment of breast cancer metastasis. Previous technologies involving anti-HGF blocking antibodies have proven difficult in inhibiting Met in vivo, particularly in carcinoma cells expressing both HGF and Met. Moreover, a systemic block of HGF or Met may cause severe damage to normal tissues, since the HGF-Met pathway is vital for normal epithelial development (Brinkmann et al. 1995). Our approach is therefore to identify tumor-associated regulatory molecules that modulate HGF expression preferentially in carcinoma cells. We have recently demonstrated that activation of two regulatory molecules, designated c-Src and Stat3, induces strong transcriptional activity of the *HGF* promoter in breast carcinoma cells. This effect involves an *HGF* promoter region that is distinct from cytokine- or estrogen-dependent regulation of *HGF* transcription in fibroblasts (Fig. 6). Sustained hyper-activation of c-Src and Stat3 has also been demonstrated to occur in invasive human breast cancer and in some instances promotes mammary tumorigenesis. In contrast, transient induction of Stat3 occurs in normal epithelial cells and promotes mammary tubulogenesis. Thus, the sustained high level of c-Src-Stat3 activity in carcinoma cells may be sufficient to induce HGF expression and autocrine activation of Met, compared with normal epithelial cells. We are therefore currently investigating whether activation of an HGF-Met autocrine loop is causally linked to the oncogenic effects of c-Src and Stat3. Approaches are in progress to identify the mechanisms by which c-Src and Stat3 regulate HGF expression in carcinoma cells and to determine whether blocking (by mutation) c-Src-Stat3-dependent HGF expression can inhibit or prevent the development of mammary tumors and metastasis. This study suggests a new signalling pathway in the transcriptional regulation of HGF expression in carcinoma cells, and could thus lead to novel strategies for the design of low molecular weight antagonists (Gambarotta et al. 1996; Epling-Burnette et al. 2001) to inhibit *HGF* gene expression preferentially in breast cancer (Fig. 8). Drugs that specifically target induction of HGF (ligand) expression in cancer cells could be used in combination with other treatments that target growth factor receptors (e.g., the use of herceptin for treatment of HER2/neu-positive breast cancer (Slamon et al. 2001)). These novel treatment strategies pro-

Fig. 8. Model of regulation of HGF expression in EMT and breast carcinomas. Paracrine stimulation by HGF of mammary epithelial cells results in tightly controlled transient activation of c-Src and Stat3 and is required for ductal morphogenesis in mammary gland development. Sustained hyper-activation of c-Src and Stat3 occurs in breast cancer by a variety of mechanisms, such as increased stimulation by upstream transducer molecules (e.g., RTKs, integrins) or mutations in regulatory oncogenes. Sustained high level of c-Src-Stat3 activation induces *HGF* transcription and HGF protein expression in epithelial cells that express Met, resulting in activation of an HGF-Met autocrine loop. These combined events promote EMT, mammary tumorigenesis, and metastasis. Thus, blocking c-Src-Stat3-dependent *HGF* transcriptional activity, by mutation or specific inhibitors, would be expected to preferentially neutralize autocrine HGF-Met activation in breast carcinoma cells.



vide an exciting new direction for inhibitor design in the treatment of breast cancer metastasis.

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Appendix III

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A novel activating function of c-Src and Stat3 on the *HGF* promoter in mammary carcinoma cells

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Running Title: Novel regulation by Stat3 of the *HGF* promoter

ABSTRACT:

In the normal breast, hepatocyte growth factor (HGF), also known as scatter factor, is expressed primarily by stromal cells, while epithelial cells express the HGF receptor, Met. Thus, epithelial cells exhibit a tight paracrine loop regulating HGF-dependent responsiveness. In invasive human breast carcinomas, HGF and Met are frequently over-expressed, thereby establishing an autocrine HGF/Met signalling pathway and promoting tumour cell invasion. However, the mechanisms leading to aberrant expression of HGF in carcinoma cells are not known. We previously demonstrated a co-operative effect of c-Src tyrosine kinase and Stat3 in the activation of *HGF* transcription in mammary carcinoma cells. In the present report, we have shown that non-malignant breast epithelial cells over-expressing activated c-Src and Stat3 exhibit increased phosphorylation of Stat3 Tyr705, *HGF* transcription, and cell scattering. Mutational analysis of the *HGF* promoter revealed a novel Stat3 binding site at nt -95, which is required for the c-Src/Stat3 co-operative effect. DNA-protein binding studies demonstrated a higher affinity binding of a Stat3-containing complex to the nt -95 site compared to other putative consensus sites at nt -110 or nt -149, respectively. Our results delineate a novel c-Src/Stat3-dependent mechanism that regulates *HGF* promoter activity. This study could define a new level of tumour specificity that might be associated with aberrant HGF expression in breast cancer. This information could lead to novel strategies for the design of small molecule antagonists to inhibit *HGF* gene expression in tumour cells with minimal effects on normal HGF/Met function.

INTRODUCTION:

Hepatocyte growth factor (HGF), also known as scatter factor, is a multifunctional cytokine. Through binding to its receptor, Met, HGF can induce cell survival, growth, morphogenesis, migration and angiogenesis (1). Both HGF (2) and Met (3) are essential for normal embryo development. In the normal breast, HGF is expressed primarily by stromal cells, while epithelial cells express Met but not HGF, thus creating a tightly controlled paracrine mechanism where localized expression of HGF regulates mammary ductal growth and differentiation (4). We (5) and others (6,7) have shown that, in contrast to what occurs in normal epithelium, HGF and Met are frequently over-expressed in invasive human breast carcinomas, as well as many other cancer types (8-13), and this high level of HGF expression has been described to be an independent predictor of poor overall survival in patients with breast cancer (14,15). These observations suggest that establishment of an autocrine HGF loop, and sustained activation of the Met signalling pathway in carcinoma cells may promote progression to invasive cancers.

Transcriptional regulation is very important in the restriction of HGF expression to mesenchymal tissues (16,17). *In vivo* analyses of *HGF* promoter regulation in transgenic mice (18), as well as *in vitro* transient transfection studies have identified important regulatory elements in the proximal promoter region of the *HGF* gene (16,19,20). Partial characterisation of this region revealed a composite element located at nt -260 to -230 from the transcriptional start site, which binds PPAR γ 1 as well as members of the NF1, AP2 and USF families to regulate *HGF* transcription. Functional studies show that NF1 and AP2 suppress the activity of the *HGF* promoter, whereas PPAR γ 1 and USF have activating functions (21-23). Additional *in vitro* studies have shown that *HGF* transcription is positively regulated by estrogen and dexamethasone, and is inhibited by TGF β (24). A negative regulatory site at nt -16 to +4 has

also been identified as being required for suppression of *HGF* transcription in normal epithelial cells (16). The DNA binding activity of this inhibitory factor is reduced by almost 80% when *HGF*-dependent proliferation is required, as occurs during liver regeneration. The mechanisms leading to aberrant *HGF* expression observed in invasive carcinomas are not known.

Increased activation of two proto-oncogenes, c-Src and Stat3, is associated with many epithelial cancers, and is linked to the expression of a number of growth factors such as vascular endothelial growth factor (25,26) and *HGF* (27). In addition, Stat3 is activated through Tyr705 phosphorylation by c-Src (28,29), and Ser727 phosphorylation by the Ras/Rac1/p38 and Jnk signalling pathways (30). In support of a role of the c-Src/Stat3 pathway in mammary tumourigenesis, we previously demonstrated a co-operative effect of c-Src and Stat3 on *HGF* transcription in the murine mammary carcinoma cell line SP1 which secretes *HGF* and expresses tyrosine-phosphorylated Met (31). We further identified a c-Src/Stat3-responsive region in the *HGF* promoter, localised between nt -254 and -70 from the transcriptional start site (27). Since both c-Src and Stat3 were already known to function as downstream effectors of the *HGF*/Met signalling pathway (32,33), the observation that these molecules can co-operatively stimulate transcription of their own activator provides a novel molecular basis for the *HGF* autocrine loop previously described in mammary carcinoma cells (31).

In the present study, we examined the mechanism of c-Src-dependent Stat3 regulation of *HGF* transcription and of the transformed phenotype of mammary epithelial cells. Stable co-expression of activated c-Src and Stat3 caused increased *HGF* transcription, concomitant with marked cell scattering in breast epithelial cells. A putative Stat3 site at nt -95 was identified, as defined by the palindromic structure 5'-TTCCC^G/_G^T/_GAA-3', which has been shown to selectively bind to protein complexes containing Stat3 (34,35). Using a mutational approach we demonstrated that the -95 consensus site is responsible for the observed co-operative effect of

c-Src and Stat3 in regulating *HGF* transcription. DNA-protein binding studies further demonstrated that this site has high affinity for a Stat3-containing complex. In this study, signalling molecules that regulate *HGF* promoter activity preferentially in carcinoma cells are identified, which could define a potentially new level of tumour specificity that might be associated with aberrant HGF expression in breast cancer.

MATERIALS AND METHODS:

Cell culture - The HC11 mammary epithelial cell line was maintained in RPMI 1640 medium (Invitrogen™) supplemented with 10% fetal bovine serum (FBS), 5 µg/ml insulin and 10 ng/ml epidermal growth factor (36). In addition, 200 mg/ml of G148 was added to the maintenance medium of HC11 cells stably transfected with pBabeY527FSrc and pRSVStat3. The SP1 tumour cell line is derived from a spontaneous poorly metastatic murine mammary intraductal adenocarcinoma and its characterisation has been described previously (31,32). The SP1 cells were maintained in RPMI 1640 medium (Invitrogen™) supplemented with 7% fetal bovine serum.

Antibodies - Rabbit polyclonal antibody against Stat3 (H-190) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Stat1, -Stat2, -Stat5a, and -Stat5b antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). A mouse monoclonal phospho-specific anti-Stat3[pY705] antibody was obtained from Cell Signalling Technology (Beverly, MA). Rabbit phospho-specific anti-c-Src[pY418] and anti-c-Src (pan) antibodies were obtained from Biosource International (Camarillo, CA) and Santa Cruz, respectively. Anti-chicken c-Src and anti-FLAG monoclonal antibodies were gifts from Dr. S. Parsons and Dr. L. Raptis, respectively.

Oligonucleotides –

Oligonucleotides used for PCR mutagenesis

HGF 5': 5'-GGGCTCGAGGGAGCCACAAGGATC-3'

HGF 3': 5'-GGGAAGCTTGAGATGCCGGGCTG-3'

HGF -149 plus: 5'-GGGCCCCGGGGCAGGAAGGGGCTGG-3'

HGF -149 minus: 5'-CCTGCCCCGGGCCCCCCC-3'

HGF -110 plus: 5'-GCCGTTCTGCAGTTTCTTACCG-3'

HGF -110 plus TM: 5'-GCCGTTCTGCAGTTTCGACGTC-3'

HGF -110 minus: 5'-GCAGAACGGCAGCCCTTTACTC-3'

HGF -95 plus: 5'-GTTTCGACGTCTAAGAGGGAG-3'

HGF -95 minus: 5'-CCCTCTTAGACGTCGAAACTG-3'

HGF -91: 5'-CCCTCGAGGTAAGAGGGAGTTC-3'

Oligonucleotides used for electrophoretic mobility shift assay

Stat3 -149F: 5'-GGGGTTGAGGAAAGG-3'

Stat3 -149R: 5'-CCCTTCCTTTCCTCAACCCC-3'

Stat3 -110F: 5'-GGGCTGTTGTAAACAGTTTCT-3'

Stat3 -110R: 5'-AGAAACTGTTTAACA-3'

Stat3 -95F: 5'-GTTTCTTACCGTAAGA-3'

Stat3 -95R: 5'-AAACTCTTACGGTAAGA-3'

Stat3 -95MF: 5'-GTTTCGACGTCTAAGA-3'

Stat3 -95MR: 5'-AAACTCTTAGACGTCGA-3'

Mutagenesis and cloning - Mutants of the HGF promoter were constructed by two-rounds of PCR mutagenesis, using the 2.7 HGF-Luc construct (27) as template. The HGF -95 minus primer was used with the HGF 5' primer to generate fragment A, whereas primers HGF 3' and HGF -95 plus were used to generate fragment B. Amplification was carried out using Vent polymerase (New England Biolabs) in an Eppendorf MicroCycler for 34 cycles. The generated fragments were purified from a 1% agarose gel and they were both used as template for one

reaction in the absence of primers followed by addition of HGF 5' and HGF 3' primers and 30

cycles amplification. The generated DNA fragment, the -95 mutant, contained a XhoI restriction site at its 5' end and a HindIII restriction site at its 3' end introduced by the HGF 5' and HGF 3' primers respectively, along with a mutation at nucleotides corresponding to positions -95 to -90 of the HGF promoter.

The -149 and -110 mutants were constructed following the same procedure, using HGF -149 plus and minus or HGF -110 plus and minus primers respectively. The triple mutant (-149, -110 and -95) was constructed by using the -149 mutant as template for the HGF 5' and HGF -110 minus primers, and the -95 mutant as template for the HGF 3' and HGF -110 plus TM primers during the first round of PCR. The introduced mutations were:

-95 site	Original Sequence	TTACCGTAA
	Mutated Sequence	GACGTCTAA
-110 site	Original Sequence	TTGTTAAA
	Mutated Sequence	CCGTTCTG
-149 site	Original Sequence	TTGAGGAA
	Mutated Sequence	CCCGGGGC

The -91 truncation mutant was created using HGF -91 and HGF 3' primers and 2.7 HGF-Luc as template. A wild type (wt) fragment was also generated by using 2.7 HGF-Luc as template, HGF 5' and HGF 3' primers and the same PCR conditions as those used for construction of the -91 mutant.

Plasmids were created by inserting the corresponding PCR fragments into the pGL2-Basic vector using standard techniques (Promega). The -149M -538 HGF-Luc, -110M -538 HGF-Luc, -95M -538 HGF-Luc and -149/-110/-95M-538 HGF-Luc constructs were generated by replacing the fragment contained between PvuII and AgeI restriction sites of the 0.5HGF-Luc construct (27) with the respective mutants generated by PCR and cut with PvuII and AgeI. -144 HGF-Luc was constructed by cutting -149M -274HGF-Luc with SmaI, followed by re-ligation.

To generate -105HGF-Luc, the -110M -274 HGF-Luc construct was digested with PstI/XhoI, treated with T4 DNA Polymerase for end blunting, and re-ligated.

For normalisation of transfection efficiency of each sample, pRL-CMV vector (Promega), which provides constitutive expression of the Renilla luciferase, was used.

Cell transfection and luciferase assay - All transfections were carried out with the LipofectAMINE Plus™ system (Invitrogen Life Technologies) according to manufacturer's instructions. HC11 cells were seeded in a 6-well plate (1×10^5 cells/well), incubated overnight in complete growth medium and transfected with 1.2 μ g of reporter plasmid, 0.2 μ g of control reporter (pRL-CMV), and up to 0.6 μ g of expression plasmids (c-Src and Stat3) as indicated. SP1 cells were seeded in a 24-well plate (1×10^4 cells/well), incubated overnight in complete growth medium and transfected with 0.4 μ g of reporter plasmid, 0.1 μ g of control reporter and up to 0.3 μ g of expression plasmids. After 48 hours transfected cells were lysed using the Passive Lysis Buffer (Promega) and the Dual-Luciferase™ Reporter (Promega) assay was performed according to manufacturer's instructions using an EG&G Berthold microplate luminometer. The Firefly luciferase activity was normalised to the corresponding CMV-Renilla luciferase activity for each well, and triplicate values for each construct were averaged. The results for each experiment were normalised to the luciferase activity of the full length HGF-Luc construct used as positive control. Normalised results from repeated experiments were pooled, and expressed as mean \pm SD. The n indicated represents the total number of wells transfected in 2 to 3 separate experiments.

Nuclear extract preparation - Cells were cultured to 80% confluency on five 100 mm tissue culture plates, harvested and washed with PBS. The washed cell pellet was resuspended in 5 ml of Cell Lysis Buffer (10 mM HEPES, 15 mM KCl, 2 mM EDTA, 0.5 mM spermidine, 0.15 mM

spermine, 0.5% Igepal, 1% dry low-fat milk, 1% trasylol, 1 mM DTT, 0.1 mM PMSF in DMSO, 1 µg/ml Leu-Pep) and incubated on ice for 5 minutes. The nuclei were isolated by spinning the cell suspension through a sucrose cushion (10 mM HEPES, 15 mM KCl, 2 mM EDTA, 0.5 mM spermidine, 0.15 mM spermine, 0.5% Nonidet P-40, 0.88 M sucrose), and resuspended in 300 µl of Nuclear Lysis Buffer (10 mM HEPES, 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 3 mM MgCl₂, 1% trasylol, 1 mM DTT, 0.1 mM PMSF in DMSO, 1 µg/ml Leu/Pep, 1 mM benzamidine). KCl was added to the resuspended nuclear solution to a final concentration of 0.55 M, and high salt extraction was performed by incubation on ice for 30 minutes with occasional mixing. The mixture was centrifuged at 15,000xg at 4°C for 20 min; the supernatant containing nuclear proteins was frozen in aliquots and stored at -70°C until used. The protein content of the nuclear extract was determined using a Micro BCA protein assay (Pierce) following manufacturer's instructions.

Probe labelling and Electrophoretic mobility shift assay - Complementary oligonucleotides were annealed by boiling together 50 µg of each oligonucleotide for 10 min and then cooling slowly to room temperature. DNA was then precipitated in ethanol, dried and resuspended in TEN₅₀ (TE pH 7.5, 50 mM NaCl) to a final concentration of 100 ng/µl. The labelling reaction was carried out by incubating 200 ng of annealed oligonucleotides with 5 U of Klenow enzyme, 1x Medium Salt Buffer (10 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT, pH 7.9), 5 mM dTTP, 5 mM dCTP, 5 mM dGTP and 20 µCi α³²P dATP (10.0 mCi/ml) for 20 min at room temperature. The reaction was chased with 5 mM dATP for 10 min. The unincorporated isotope was removed by passing the solution through a G50 column.

The binding reaction for EMSA was performed by incubating the nuclear extract with 1µg poly (dI-dC), 1x Bandshift Binding Buffer (10 mM HEPES, 20 mM KCl, 1.5 mM MgCl₂,

2% Ficoll, 5 mM DTT), 10 µl BSA and 1 ng of ^{32}P -labelled probe on ice for 20 min. A 6% acrylamide gel was pre-run in 0.25x TBE for 30 min at 100 V; the samples were then loaded and electrophoresis was carried out at 150 V for 2.5 h at 4°C. The gel was fixed in 7% acetic acid, 40% ethanol for 20 min, vacuum dried on 3 MM Watmann paper and exposed to autoradiograph and PhosphorImager screen for analysis. For supershift experiments, nuclear extracts were incubated with 1 µg of the indicated antibody for 30 min on ice, prior to the binding reaction.

Indirect immunofluorescence - Cells were plated overnight on cover slips, rinsed three times in pre-warmed PBS with 0.1 µM CaCl_2 and 0.1 µM MgCl_2 , and fixed for 20 min in 3% paraformaldehyde in PBS. Cells were permeabilised by incubation for 5 min in 0.2% Triton X-100 in PBS, rinsed three times, and incubated for 10 min in 50 mM NH_4Cl in PBS. Cells were incubated with anti-chicken c-Src antibody for 45 min, washed three times in PBS, and incubated with the appropriate secondary antibody. All antibody concentrations were predetermined to yield optimal signal noise ratios. Preparations were observed using a Zeiss Axiovert inverted microscope in the Protein Discovery and Function Facility at Queen's University. Image acquisitions were processed using Adobe Photoshop software.

Western blotting - Cells were grown to confluence, rinsed with PBS, and lysed in a RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EGTA, 1% NP-40, 0.1% SDS, 1 mM Na_3VO_4 , 50 mM NaF, 2 µg/ml aprotinin, 2 µg/ml leupetin, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged for 10 min at 14,000 rpm in an IEC/Micromax centrifuge, and supernatants were subjected to protein determination using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Equal protein amounts of each cell lysate were subjected to 8% SDS-PAGE under reducing conditions (with 2.5% β -2 mercaptoethanol), and transferred to

nitrocellulose membranes. Separate membranes were probed with the indicated primary antibodies. Immune complexes were detected using horseradish peroxidase-labelled donkey anti-rabbit or anti-mouse IgG, followed by chemiluminescence (NENTM Life Science Products Inc., Boston, MA).

RESULTS:

Over-expression of both activated c-Src and Stat3 in mammary epithelial cells causes increased phosphorylation of Stat 3 Tyr705 and promotes cell scattering - We previously demonstrated co-operativity of c-Src and Stat3 in the regulation of *HGF* transcription in mammary epithelial and carcinoma cell lines (27). This finding prompted us to determine whether c-Src-dependent activation of Stat3 correlates with cell scattering in epithelial cells. A non-malignant mammary epithelial cell line, HC11, was stably transfected with an activated chicken c-Src mutant (clone C11), or FLAG-tagged Stat3 (clone C20), or activated c-Src + Stat3 (clone C20/C2). Expression of the corresponding activated c-Src and Stat3 proteins was confirmed by western blotting analysis (Fig. 1A). We first determined the effect of combined over-expression of c-Src and Stat3 on cell scattering of HC11 epithelial cells, using F-actin staining to assess cell shape and filopodia extensions. Untransfected HC11 cells, or cells expressing Stat3 or activated c-Src alone, grew in rounded cell islets with no scattering detected (Fig. 1C-1H). However, marked filopodia extensions and scattering was observed in cells expressing both activated c-Src and Stat3 (Fig. 1I and 1J). Expression of activated c-Src alone, but not Stat3, caused marginal cell spreading with no scattering (Fig. 1E and 1F). In contrast to HC11 cells, we have previously shown that expression of activated c-Src alone in SP1 carcinoma cells is sufficient to induce cell scattering (17). Thus activated c-Src acts co-operatively with Stat3 to induce cell scattering in nonmalignant epithelial cells.

To assess the status of c-Src-dependent activation of Stat3 in the above cell lines, we determined the status of Stat3 Tyr705 phosphorylation, which is induced by c-Src and promotes Stat3 dimerization (28,29) (Fig. 2A). Clone C20/C2, over-expressing both activated c-Src and Stat3, showed a high level of Stat3 Tyr705 phosphorylation, whereas over-expression of activated c-Src or Stat3 alone caused only a marginal effect. HC11 clones expressing activated c-

Src (clones C11 and C20/C2) showed enhanced phosphorylation of c-Src Tyr418 compared to untransfected cells (Fig. 2B), indicating increased autophosphorylation of the kinase domain of c-Src in these cells. In contrast to HC11 cells, untransfected SP1 carcinoma cells showed an intermediate level of spontaneous phosphorylation of Stat3 Tyr705. Thus over-expression of both activated c-Src and Stat3 results in increased phosphorylation of Stat3 Tyr705 in HC11 cells, correlating with increased cell scattering.

The nt -254 to -70 region of the HGF promoter is required for c-Src/Stat3-induced HGF transcription in epithelial cells - The c-Src/Stat3 responsive region of the *HGF* promoter, located between nt -254 and -70 in relation to the start of transcription site, has been shown to be required for the observed increase in *HGF* transcription in response to activated c-Src and Stat3 in the malignant cell line SP1 (27). To assess the role that this region plays in the response of nonmalignant cells to c-Src and Stat3, the mammary epithelial cell line HC11 was co-transfected with reporter constructs containing various deletions of the *HGF* promoter linked to the Firefly luciferase gene (27), and a combination of vectors expressing Stat3 or the activated form of c-Src (Fig. 3). Neither the expression of c-Src nor Stat3 alone produced a significant effect on *HGF* transcription, however their combined expression resulted in a strong induction of *HGF* promoter activity. Furthermore, deletion mutants of the *HGF* promoter lacking the nt -254 to -70 region showed no response to over-expression of c-Src and Stat3, similar to what has been described in malignant SP1 cells (27). These results indicate that the nt -254 to -70 region of the *HGF* promoter is required for the observed increase in *HGF* transcription in response to c-Src and Stat3 in epithelial cells.

c-Src/Stat3 responsiveness of the HGF promoter maps to the nt -105 to -91 region -

Examination of the c-Src/Stat3 responsive region of the *HGF* promoter revealed three putative Stat3-binding consensus sites at nt -149, -110 and -95. To determine the possible role that these sites play in the observed *HGF* promoter activity, the transcriptional activities of truncated forms of the *HGF* luciferase reporter lacking only the -149 site (-144 HGF-Luc), both -149 and -110 sites (-105 HGF-Luc), or all three Stat3 binding sites (-91 HGF-Luc), were compared to that of the previously described -274 HGF-Luc and -70 HGF-Luc constructs (Fig. 4). Each reporter construct was transfected into HC11 cells alone, or in combination with activated c-Src and Stat3 plasmids. Expression of activated c-Src and Stat3 induced activation of -274 HGF-Luc, -144 HGF-Luc and -105 HGF-Luc, but no increase in the -91 HGF-Luc or -70 HGF-Luc transfected cells was observed. The -105 HGF-Luc construct lacks over 80% of the originally identified c-Src/Stat3 responsive region, including two of the consensus Stat3 binding sites, -149 and -110. Interestingly, promoter activity was abolished when an additional 15 base pairs were deleted (compare -105 HGF-luc to -95 HGF-luc). Similar results were obtained when the same deletion mutant HGF reporter plasmids were expressed in SP1 cells (data not shown). Together, these results further narrow the sequence that is required for HGF responsiveness to c-Src and Stat3, and strongly suggest that of the three sites, only the one located at nt -95 is required for transcriptional activation in response to c-Src and Stat3.

The -95 Stat3 consensus site is required and sufficient for c-Src/Stat3 responsiveness of the

HGF promoter in epithelial cells - To further analyse the role of each putative Stat3 consensus site, reporter plasmids containing the -274 HGF-Luc sequence mutated at each site (-149M -274 HGF-Luc, -110M -274 HGF-Luc and -95M -274 HGF-Luc) were constructed. The mutagenesis

strategy involved replacing the first two nucleotides of each consensus Stat3 binding sequence

(TT) with C or G. Given that the TT element has been shown to play a critical role during Stat3 binding to DNA, such mutations should abolish Stat3-DNA interactions at the sites of interest. Activity of the mutants was compared with that of the wild type (wt) -274 HGF-Luc (Fig. 5). Each reporter construct was transfected into SP1 cells alone, or combined with Stat3, c-Src or a combination of both plasmids. Expression of the c-Src/Stat3 plasmids induced activation of constructs mutated at positions -149 and -110, similar to what was observed in the wild type -274 HGF-Luc construct whereas no such induction was observed in the -95M -274 HGF-Luc transfected cells. To further confirm the apparent dominance of the -95 Stat3 binding site in regulating HGF promoter activity, an additional mutant was constructed in which all three consensus Stat3 binding sites were mutated (-149M/-110M/-95M -274 HGF-Luc). The triple mutant response to co-expression of c-Src and Stat3 resembled that of -95M -274 HGF-Luc.

The above results were reproduced in HC11 cells, using the -538 HGF-Luc reporter, which has been shown to display expression activity comparable to that of the full-length *HGF* promoter (27). As was observed in -274 HGF-Luc mutants, co-expression of activated c-Src and Stat3 induced activation of wild type -538 HGF-Luc, -149M -538HGF-Luc and -110M -538 HGF-Luc, but not in -95M -538 HGF-Luc or the triple mutant (-149M/-110M/-95M -538HGF-Luc) (Fig. 6). These observations support the critical role of the Stat3 consensus site located at position -95 of the *HGF* promoter that was apparent from the analysis of truncated constructs. The fact that both the -149M and the -110M mutant constructs retained their capacity to respond to c-Src/Stat3 stimulation indicates that the interference with Stat3 binding to these two elements does not prevent activation of *HGF* transcription in response to Stat3, and suggests that, under these conditions, they do not play a significant role as Stat3 binding sites during the process of *HGF* transcription activation.

A nuclear protein complex exhibits high affinity binding to the -95 consensus site of the HGF promoter - Our demonstration of high levels of Stat3 Tyr705 phosphorylation in C20/C2 cells (expressing activated c-Src and Stat3) compared to wild type HC11 cells (Fig. 2) suggests the presence of high nuclear levels of the activated form of Stat3, which should have the capacity of binding to DNA. Electrophoretic mobility shift assays (EMSA) were therefore performed to examine the DNA binding affinity of nuclear protein extracts from SP1 carcinoma, wild type HC11, and HC11 cells with stably integrated activated c-Src and Stat3 (C20/C2), towards the three different Stat3 consensus sites. To detect protein complex binding, ³²P-labelled oligonucleotide probes with DNA sequences corresponding to the original and the mutated -95 consensus sequence were incubated with nuclear extracts from SP1, HC11 and HC11 C20/C2 cells (Fig. 7A). A band shift indicative of DNA-protein interaction was observed when the wild type sequence was used as probe, but not when the mutated sequence was used, suggesting that the point mutation disrupted the putative Stat3 binding site and abolished interaction of the protein with the *HGF* promoter. Interestingly, the band shift assay revealed a difference between HC11 C20/C2 and the other two cell lines, as it showed three bands versus only two bands seen in samples from SP1 and HC11 cells.

In order to confirm the presence of Stat3 in the observed protein-DNA complexes, nuclear extracts were preincubated with anti-Stat3 antibody prior to the addition of ³²P-labelled oligonucleotides corresponding to the -149, -110 and -95 consensus Stat3 binding sites. The assay showed weak binding activity when the -149 and -110 probes were used, and the complexes formed were not affected in their mobility by anti-Stat3 antibody, supporting the dominant role of the -95 site (Fig. 8). Interestingly, the supershift assay identified Stat3 in complexes formed between the -95 ³²P-oligonucleotide and nuclear extracts from HC11 C20/C2 cells, but no additional band retardation was seen when extracts from SP1 or HC11 cells were

used instead. This finding could be attributed to the higher level of Stat3 activation in HC11 C20/C2 cells (Fig. 2A).

Since it is known that other members of the Stat family (Stat1, Stat5a and Stat5b) may also bind to the Stat3 consensus sites, albeit at lower levels, antibodies against specific Stat proteins were used in supershift experiments to determine the composition of the DNA binding complex. When nuclear extracts from HC11 C20/C2 cells were preincubated with antibodies against Stat1, Stat2, Stat3, Stat5a and Stat5b prior to the addition of radiolabelled probe, only the anti-Stat3 antibody produced a supershift band (Fig. 7B). The major complex (lower band) binding to the -95 consensus site showed no supershift indicating that it is not a Stat protein.

To further characterise the Stat3 binding affinity of the -95 site, competition assays were performed using 5-, 10- and 50-fold excess of unlabelled probe. It was observed that protein binding to the -95 site was abolished by the presence of excess of unlabelled -95 fragments, but the addition of corresponding amounts of -149 and -110 unlabelled probes did not produce a comparable effect, nor did an excess of the mutated -95 sequence (Fig. 9). This finding indicates that the affinity of the -149 and -110 sites for Stat3 is significantly lower than that of the -95 site, and is in agreement with the transcription studies. The competition assays also showed that another transcription factor binds the -95 sequence, and this binding is highly specific. Furthermore, unlike Stat3 (Fig. 7), this factor is present in sufficient amounts in all three cell lines studied, and it binds to the -95 sequence with higher affinity than Stat3 (Fig. 9). Mutation of the -95 site significantly reduces the binding of this unidentified transcription factor; however it does not abolish binding completely, indicating that the binding sequences of both proteins share some nucleotides but are not identical.

DISCUSSION:

Whereas HGF expression is under tight negative regulation in breast epithelial cells (4), increased expression of HGF and its receptor Met frequently occurs in breast carcinomas, and is often associated with tumour invasiveness and metastatic behaviour (5,31,37,38). The molecular mechanisms responsible for increased HGF expression in carcinoma cells remain largely unknown. However, some insight into this phenomenon was previously provided by our laboratory in the characterisation of a c-Src/Stat3-responsive region in the *HGF* promoter, localised between nucleotides -254 and -70 from the transcriptional start site, which is required for c-Src/Stat3-induced *HGF* transcription in carcinoma cells (27). In the present study, we show that c-Src and Stat3 can act co-operatively to induce *HGF* transcription in mammary epithelial cells, and that the c-Src/Stat3 responsive region plays a critical role during this activation. We have further identified a novel Stat3 binding site at nt -95 of the *HGF* promoter. These observations suggest that the lack of c-Src kinase activity and the low level of activated Stat3 may be responsible for limiting *HGF* transcription in epithelial cells of the breast. Thus, overriding the tight repression of *HGF* transcription would require at least two events: increased activation of c-Src and over-expression of Stat3, with the latter being phosphorylated at Tyr705 and activated by the former (29).

Three consensus binding sites for Stat3, located at positions -149, -110 and -95, are localised in the c-Src/Stat3 responsive region of the *HGF* promoter (39). Using truncated constructs of the *HGF* promoter, we further narrowed the previously described c-Src/Stat3 responsive region to a 15 bp sequence between nucleotides -105 and -91 from the start of transcription site, which contains only one of the three originally identified putative Stat3-binding sequences at nt -95. Luciferase assays using *HGF* promoter constructs mutated at each of the three consensus Stat3 binding sites at nt -149, -110 and -95 further confirmed the critical

role of the -95 site in inducing transcription of *HGF* in both malignant and nonmalignant mammary cells. It was also observed that while basal *HGF* transcription is not affected by deletion of the c-Src/Stat3 responsive region or mutation of the nt -95 site, induction of transcription in response to Stat3 appears to be mediated exclusively by this element. These results define a novel regulatory site on the *HGF* promoter, and establish a direct role for c-Src/Stat3 in *HGF*-mediated tumourigenesis. Since there is 99% homology among the mouse, rat and human *HGF* promoter sequences between nucleotides -150 and +1, and the regulatory site described here is completely conserved among these species, the regulation of *HGF* expression by Stat3 through the -95 consensus site is probably also conserved in human cells (39).

To confirm the role of Stat3 in the observed induction of *HGF* transcription, we studied the protein-DNA complexes formed at the -95 site. Electrophoretic mobility shift assays revealed the formation of protein-DNA complexes when nuclear extracts were incubated with the ³²P-labelled -95 oligonucleotide, which was almost completely abolished by mutation of the -95 Stat3 consensus sequence. Similar complexes, albeit significantly weaker, were also identified at the -149 and -110 Stat3 consensus sites. Preincubation of nuclear extracts with anti-Stat3 antibody resulted in a retarded gel migration of the largest of the observed protein-DNA complexes formed at the -95 site, when HC11 C20/C2 nuclear extracts were used. Preincubation of SP1 and HC11 nuclear extracts with anti-Stat3 antibody produced no detectable further retardation of any of the complexes formed. Competition assays using 5-, 10- and 50-fold excess of unlabelled oligonucleotides confirmed a higher affinity of Stat3 for the -95 sequence, compared to the -110 and -149 sites.

These observations underline the critical role that over-expression of c-Src and Stat3 plays in activation of *HGF* transcription, while exposing a possible control mechanism that

restricts the over-expression of *HGF* in response to Stat3 in normal cells. As shown by the transcriptional studies, both cell lines must over-express Stat3 to exhibit an increase in *HGF* transcription, and the over-expressed Stat3 requires co-expression of activated c-Src in order to become activated through phosphorylation at Tyr705. This is true despite the fact that SP1 cells normally express high endogenous levels of Stat3, a characteristic that could account for the induction of *HGF* transcription observed in these cells after transfection with activated c-Src.

The above experiments also identified a protein-DNA complex at the -95 site in all three cell lines, that does not react with anti-Stat3 antibodies in supershift assays. To further characterise the non-Stat3 protein-DNA complexes, additional supershift studies were performed using antibodies against specific Stat proteins. The results excluded Stat1, Stat2, Stat5a and Stat5b as components of these protein-DNA complexes. Tests for Stat4 and Stat6 were not performed, but it is unlikely that these are involved in this phenomenon since they are not normally active in mammary tissue (40). The observed protein-DNA interaction was found to be very strong in competition assays similar to those used to test Stat3 affinity for the same fragment, which suggests that this non-Stat3 molecule may somehow be involved in regulating *HGF* transcription at the -95 site. A growing number of proteins capable of interacting with Stat3 and modulating its signalling has been identified, and several levels of regulation are proposed (41). Further studies will determine how these molecules interact with c-Src/Stat3 in *HGF* transcription regulation.

The mechanisms that lead to over-expression and activation of Stat3 and c-Src in tumour cells are not clearly known, but most likely involve multiple oncogenic changes. Both molecules are critical downstream effectors of Met and are required for HGF-mediated malignant phenotypes (32,33,42). The present study shows that c-Src and Stat3 can also act as upstream regulators of HGF expression, and could therefore lead to establishment of an HGF autocrine

loop, signal amplification, and an invasive phenotype. Evidence from our study supports the hypothesis that aberrant activation of c-Src and over-expression of Stat3, acquired during epithelial-mesenchymal transition of epithelial cells, could overcome the mechanisms repressing *HGF* transcription. In this model, Stat3 is activated by c-Src through phosphorylation of Tyr705 of Stat3, although concomitant phosphorylation of Ser727 of Stat3 by kinases such as Rac1 and p38 (29) is most likely required for optimal Stat3 activity. This activation process is distinct from mechanisms regulating basal levels of HGF expression, which involve Stat3-independent pathways (eg estrogen receptor or IL-6) (17). Since c-Src/Stat3-dependent activation of *HGF* transcription is preferentially associated with epithelial-mesenchymal transition, this signalling pathway may be an effective target for disruption of autocrine HGF loops and abrogation of metastasis.

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FIGURE LEGENDS:

Fig. 1. Co-expression of activated c-Src and Stat3 stimulates scattering of HC11 mammary epithelial cells.

Panel A) A mouse mammary epithelial cell line (HC11) was transfected with an activated mutant of chicken c-Src (C11), FLAG-tagged Stat3 (C20), or activated c-Src + Stat3 (C20/C2). Cells were grown to 50% confluence and cell lysates were made. Equal protein amounts were subjected to 8% reducing SDS-PAGE, and proteins were transferred to PVDF membranes. Western blotting of separate membranes was carried out with anti-chicken c-Src (upper blot) and anti-pan-c-Src (lower blot) antibodies. Immune complexes were visualized using chemiluminescence as described in the Materials and Methods.

Panel B) Western blotting of parallel blots of the above samples was carried out with anti-FLAG (upper panel) and anti-pan (lower blot) Stat3 antibodies.

Panels C-J) The parental HC11 cells and derived cell clones were grown in monolayer cultures on glass coverslips, fixed, and double stained with anti-chicken c-Src (specific for the activated c-Src protein) detected by Alexa-488-conjugated secondary antibody (GREEN) (C,E,G,H), and TRITC-phalloidin (RED) (D,F,H,J), as described in "Materials and Methods". Results are representative of two experiments.

Fig. 2. Co-expression of activated c-Src and Stat3 in HC11 cells induces high levels of Stat3 Tyr705 phosphorylation.

Panel A) Cell lysates were made from SP1, HC11 and HC11 C20/C2 cells (see Fig. 1 for designations), and equal protein amounts from each cell line were subjected to reducing 7% SDS-PAGE. The proteins were transferred onto PVDF membranes and separate blots were

probed with anti-Stat3[pY705] (upper blot) or anti-Stat3 (pan) (lower blot) antibodies. Immune complexes were visualized using chemiluminescence as described in the Materials and Methods.

Panel B) Parallel blots were probed with anti-c-Src[pY418] (upper blot) or anti-c-Src (pan) (lower blot) antibodies.

Fig. 3. The nt -254 to -70 region of the HGF promoter is required for responsiveness of HGF transcription to c-Src and Stat3 in HC11 cells.

The 2.7 kb HGF Firefly luciferase reporter (2.7 HGF-Luc), or reporter constructs containing the indicated deletions of the *HGF* promoter were co-transfected into HC11 cells with activated c-Src, Stat3, c-Src/Stat3 or an empty vector (control). A Renilla luciferase expression plasmid (pRL-CMV) was co-transfected in each group for normalisation to account for differences in transfection efficiency. After 48 h incubation, cells were lysed and luciferase activity for each sample was determined and normalised. Values represent mean \pm SD of triplicate samples and are representative of two experiments.

Fig. 4. The nt -105 to -91 region of the HGF promoter is required for responsiveness of HGF transcription to c-Src and Stat3 in HC11 cells.

The -274 HGF- Luc reporter, or truncated reporter constructs containing the indicated deletions of the *HGF* promoter, were co-transfected into HC11 cells with activated c-Src, Stat3, c-Src/Stat3 or an empty vector (control). After 48 h incubation, cells were lysed and luciferase activity for each sample was determined and normalised. Values represent mean \pm SD of nine samples.

Fig. 5. Mutation of the -95 Stat3 binding site inhibits the responsiveness of HGF transcription to c-Src and Stat3 in SP1 carcinoma cells.

Panel A) Various -274 HGF-Luc constructs mutated at the consensus Stat3 binding sites as indicated were co-transfected into SP1 cells with activated c-Src, Stat3, c-Src/Stat3 or an empty vector (control). Wild type -274 HGF-Luc and -70 HGF-Luc were used as positive and negative controls, respectively. After 48 h incubation, cells were lysed and luciferase activity for each sample was determined and normalised. Values represent mean \pm SD of six samples.

Panel B) Mutations introduced at each consensus Stat3 binding site are shown.

Fig. 6. Mutation of the -95 Stat3 binding site inhibits the responsiveness of HGF transcription to c-Src and Stat3 in HC11 cells.

Various -538 HGF-Luc constructs mutated at the consensus Stat3 binding sites as indicated were co-transfected into HC11 cells with activated c-Src, Stat3, c-Src/Stat3 or an empty vector (control), as indicated. Wild type -538 HGF-Luc and -70 HGF-Luc were used as positive and negative controls, respectively. After 48 h incubation, cells were lysed and luciferase activity for each sample was determined and normalised. Values represent mean \pm SD of eight samples.

Fig. 7. Protein-DNA complexes form at the -95 consensus site.

Panel A) Equal protein amounts of nuclear extracts from SP1, HC11 and HC11 C20/C2 cells were used for EMSA binding studies with radiolabelled oligonucleotides containing either the -95 or the -95M sequence, as described in Materials and Methods. The arrow indicates the position of the protein-DNA complex.

Panel B) Nuclear extracts from HC11 C20/C2 cells were incubated with the indicated anti-Stat antibodies on ice for 30 min prior to EMSA analysis. Positions of the Stat3-DNA and the antibody-Stat3-DNA complexes are indicated.

Fig. 8. Stat3 forms part of the protein-DNA complex at the -95 consensus site.

Nuclear extracts from SP1, HC11 and HC11 C20/C2 cells were incubated with anti-Stat3 antibody on ice for 30 min prior to EMSA analysis. After incubation with ³²P-labelled -95, -110 or -149 oligonucleotides, the reaction was subjected to non-denaturing PAGE, as described in Materials and Methods. The supershifted band representing the antibody-Stat3-DNA complex is indicated.

Fig. 9. The -95 site binds Stat3 with greater affinity than the -149 and -110 sites.

Panel A) Nuclear extracts from HC11 C20/C2 cells were incubated for 30 min with ³²P labelled -95 sequence and a 5-, 10- or 50-fold excess of unlabelled -95, -95M, -110 or -149 oligonucleotides, and subjected to EMSA analysis. The asterisk (*) indicates the Stat3 protein-DNA complex.

Panel B) Formation of protein-DNA complexes in SP1 and HC11 cells was assessed as in Panel A.

Figure 1

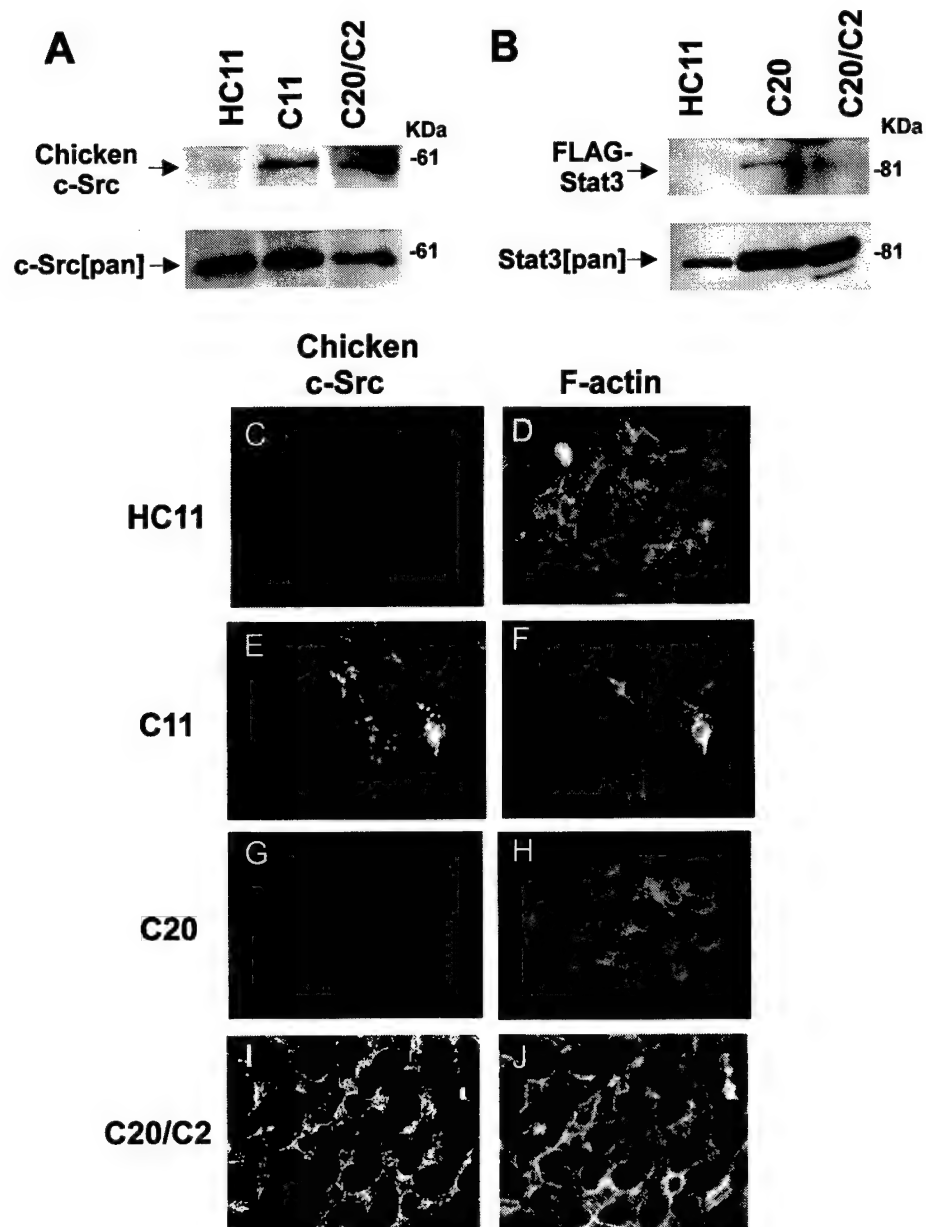


Figure 2

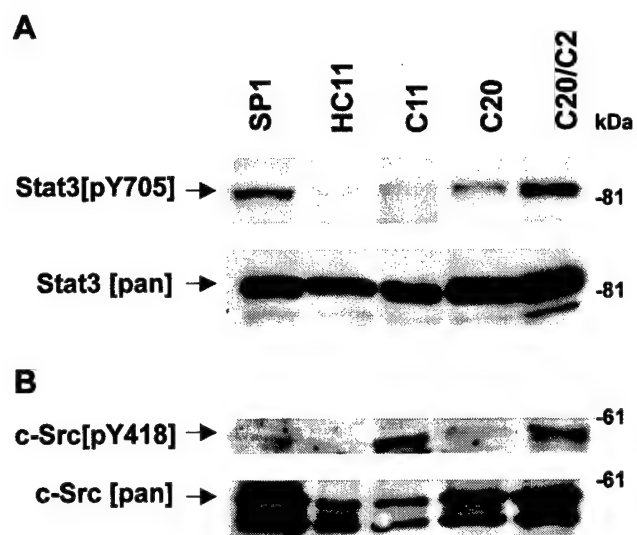


Figure 3

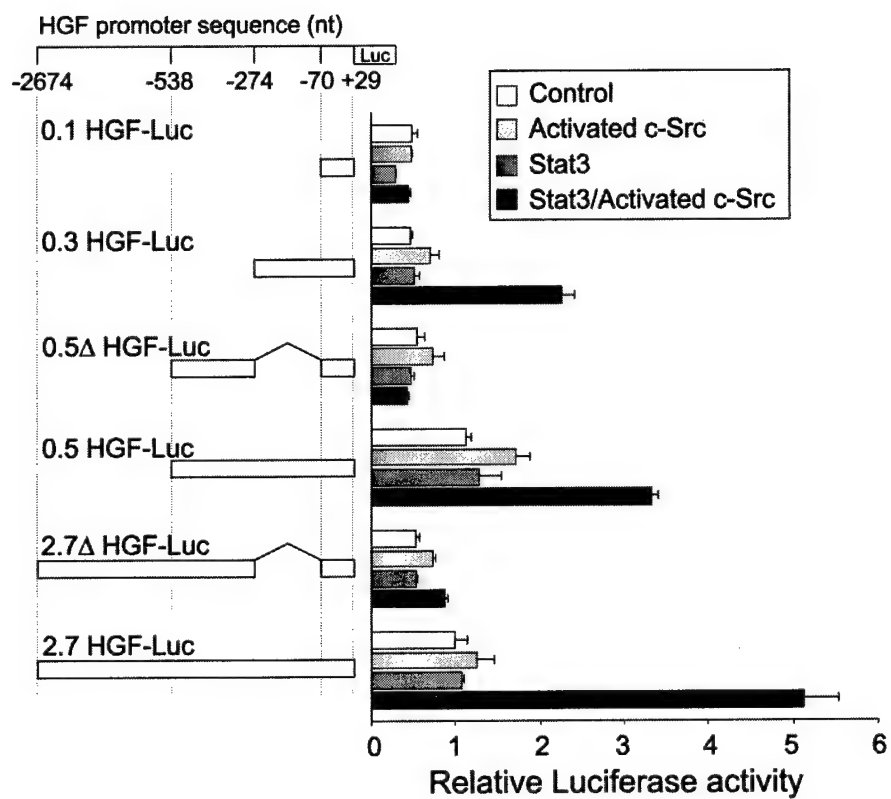


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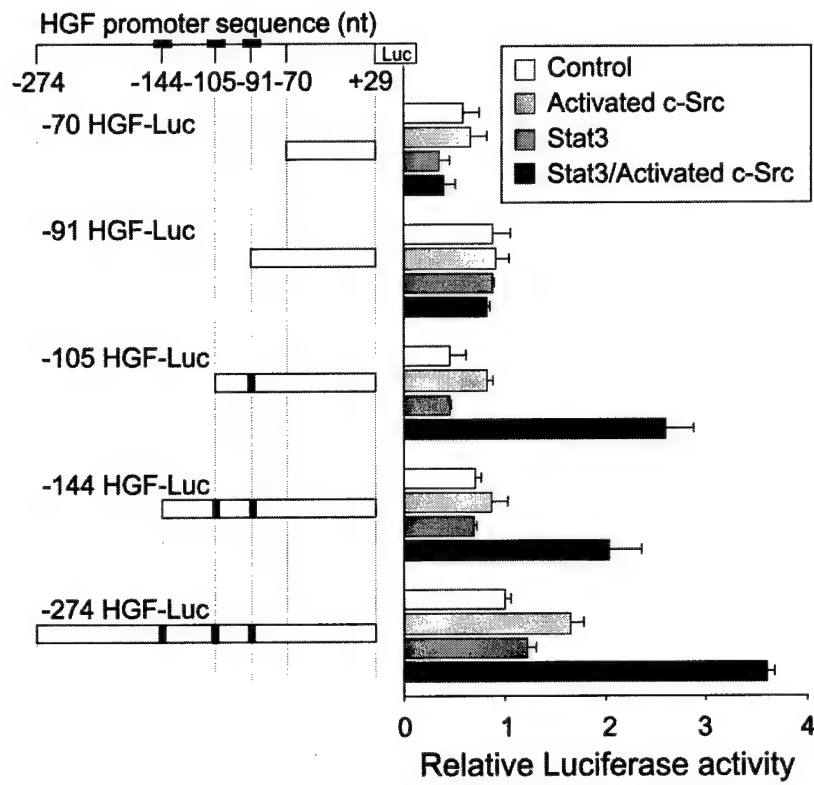
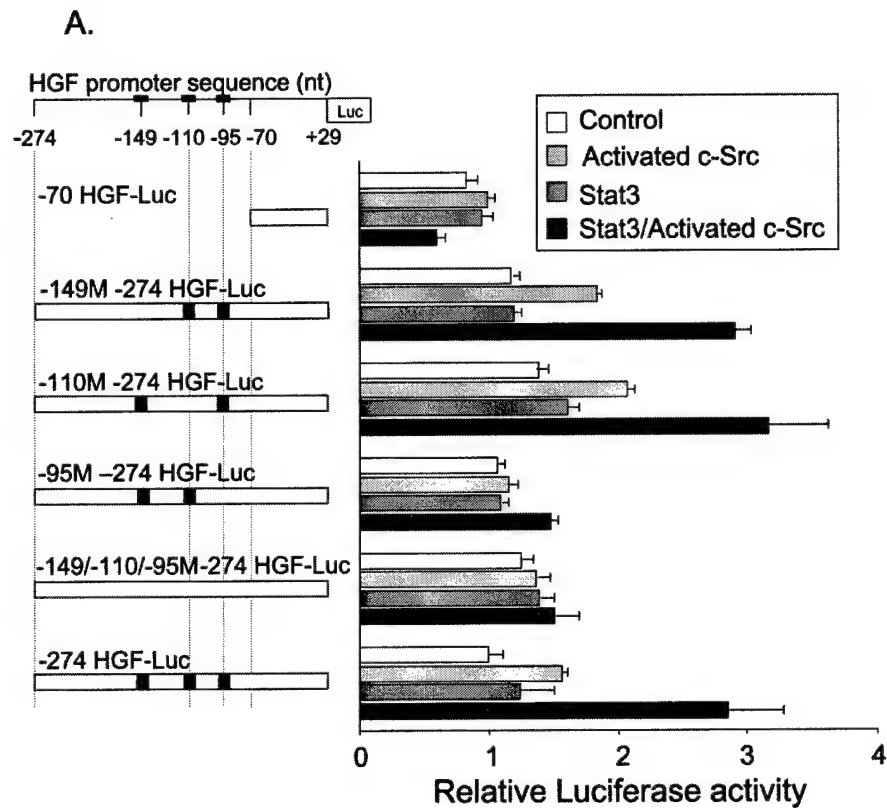


Figure 5



B.

Consensus Stat3 binding site:		TTCCC ^{GG} _{GT} AA
-95 site	Original Sequence	TTACCGTAA
	Mutated Sequence	GACGTCTAA
-110 site	Original Sequence	TTGTT AAA
	Mutated Sequence	CCGTT CTG
-149 site	Original Sequence	TTGAG GAA
	Mutated Sequence	CCCGG GGC

Figure 6

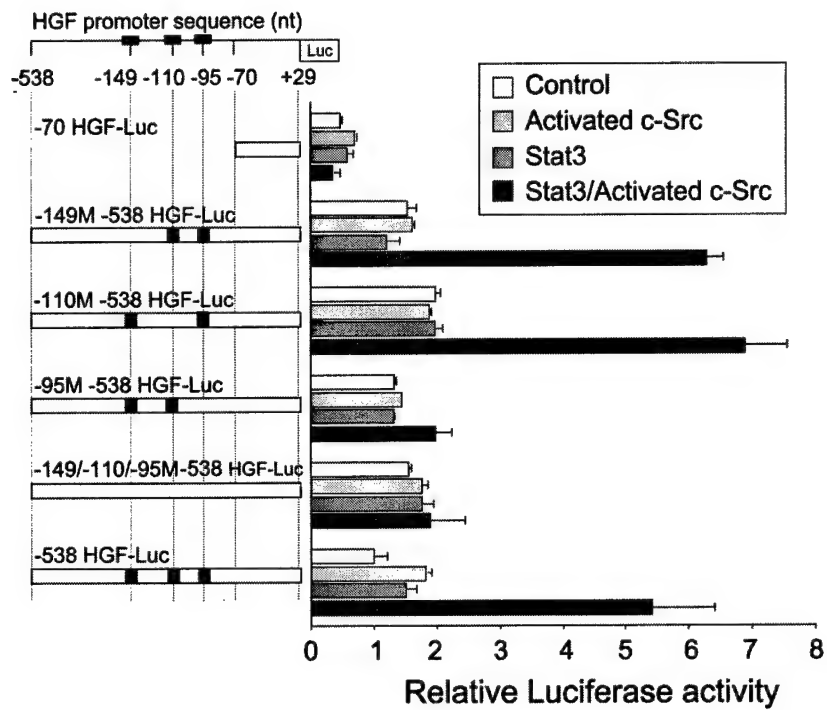


Figure 7

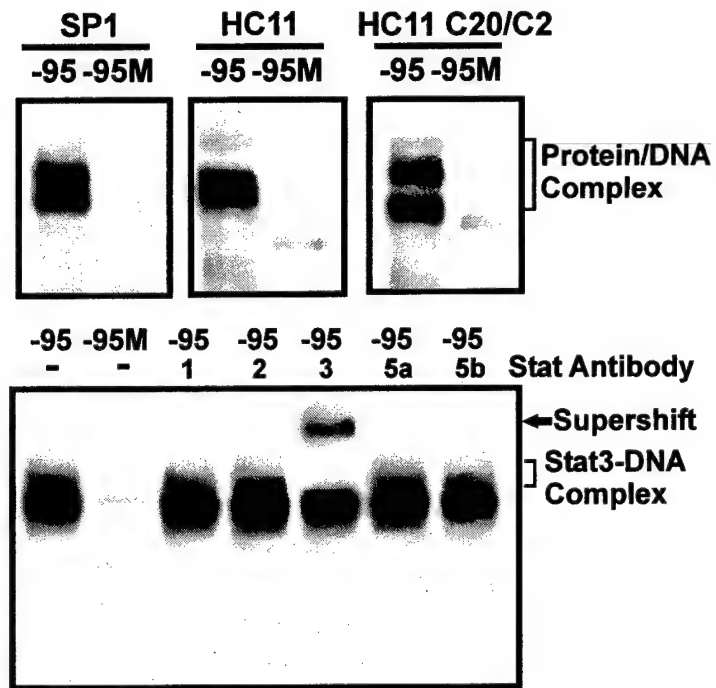


Figure 8

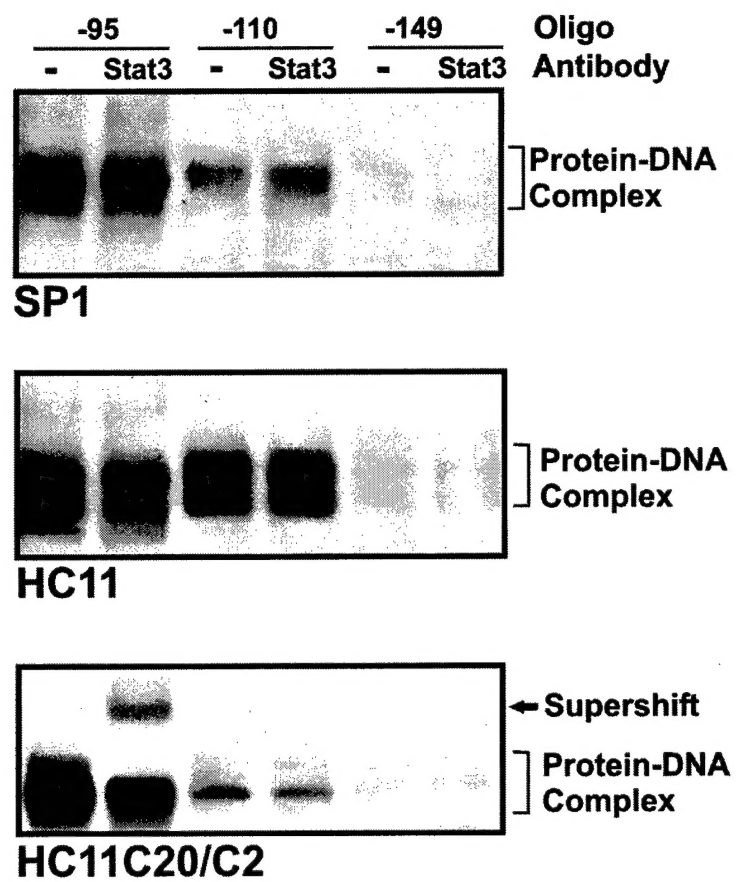
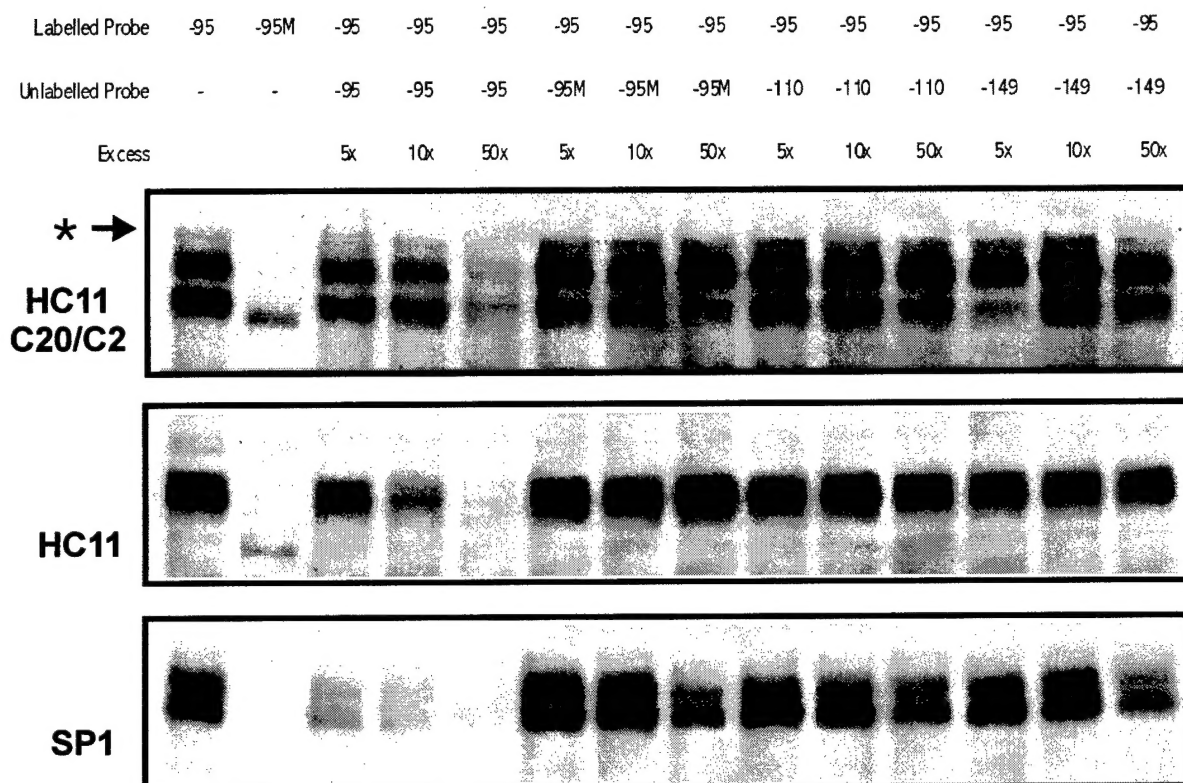


Figure 9



Appendix IV

A novel activating function of Stat3 on the *HGF* promoter in breast cancer

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Hepatocyte Growth Factor (HGF), also known as Scatter Factor, is a cytokine required for normal breast development. In the normal breast, it is expressed only by stromal cells, while epithelial cells express the HGF receptor – Met, creating a tight control mechanism for HGF-dependent growth. In contrast to what is observed in normal epithelium, HGF and Met are frequently over-expressed in invasive breast carcinomas, and this high expression has been described to be an independent predictor of poor survival in patients with breast cancer. These observations suggest that establishment of an autocrine HGF loop and sustained activation of the Met signalling pathway in carcinoma cells may promote tumour invasion, but the mechanisms leading to aberrant expression of HGF in carcinoma cells are not known. A number of signalling molecules, such as c-Src, Grb2/Ras and PI3-kinase have been shown to be part of the HGF/Met signalling pathway. It is also known that c-Src is an important requirement for mammary tumourigenesis, although its activation is not sufficient to induce malignant transformation. Using deletion mutants of the *HGF* promoter, a region (between –254 and –70 bp) was identified which is responsive to increased c-Src activity in malignant breast carcinoma cells. Three putative consensus binding sites for Stat3 were identified in this region; we therefore examined the role of Stat3 in c-Src-dependent regulation of *HGF* transcription. A strong cooperative activation of *HGF* transcription was observed after over-expressing Stat3 and activated c-Src in both malignant and non-malignant breast epithelial cells. In addition, co-expression of Stat3 and activated c-Src caused marked cell scattering in breast epithelial cells. Using a mutational approach we identified a Stat3 binding site at the –95 bp position of the *HGF* promoter, which is responsible for this cooperative effect. A point mutation at this position caused a complete inhibition of responsiveness of the *HGF* promoter to Stat3/c-Src. In this study, signalling molecules that regulate *HGF* promoter activity preferentially in carcinoma cells are identified, which could define a potentially new level of tumour specificity that might be associated with aberrant HGF expression in breast cancer. This information could lead to novel strategies for design of small molecule antagonists, such as derivative peptides or decoy oligos, to inhibit *HGF* gene expression in tumour cells with minimal effects on normal HGF/Met function.

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Appendix V

CO-OPERATIVE EFFECT OF C-SRC AND STAT3 IN STIMULATING HGF EXPRESSION AND SCATTERING IN MAMMARY CARCINOMA CELLS

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We have previously shown co-expression of hepatocyte growth factor (HGF) and its receptor Met in the invasive tumor front of human breast carcinomas. We have also demonstrated secretion of HGF, constitutive activation of Met, and increased invasion in a mouse breast carcinoma cell line, SP1. In contrast, HGF expression is suppressed in normal epithelial cells. These observations suggest the presence of an HGF autocrine loop in some breast carcinoma cells, which confers increased survival, growth, and invasiveness during tumor progression and metastasis. c-Src tyrosine kinase, which is critical in regulating the expression of many genes, is activated in SP1 carcinoma cells, as well as in most human breast cancers. We therefore examined the role of c-Src kinase in HGF expression in breast carcinoma cells.

Our approach was to use both pharmacological and mutational approaches to modulate c-Src activity in carcinoma cells. Expression of an activated c-Src mutant in SP1 cells increased transcription from the *HGF* promoter and expression of HGF mRNA and protein, while a dominant negative c-Src mutant had the opposite effect. An initial deletion analysis showed that a region between -254 and -70 base pairs was required for c-Src responsiveness of the *HGF* promoter. This region contains three putative consensus sequences (at -95, -110 and -149 bps) for the signal transducer and activator of transcription-3 (Stat3) factor. We therefore hypothesized that c-Src co-operates with Stat3 in regulating HGF expression and scattering function.

Co-expression of activated c-Src and Stat3 synergistically induced strong *HGF* promoter activity in mammary carcinoma cells and nonmalignant epithelial cells. Further deletion analysis mapped c-Src/Stat3 responsiveness of the *HGF* promoter to a region (-104 to -70 bp), which included the putative -95 Stat3 consensus site. In addition, over-expression of c-Src and Stat3 causes marked scattering of mammary epithelial cells. Collectively, our data indicate a cooperative effect of c-Src kinase and Stat3 in the activation of *HGF* transcription and protein expression in breast carcinoma cells. This process may be important in overriding the strong suppression of HGF expression in nonmalignant epithelium, and may be a key step in the early stage tumorigenesis.

Information from this study could lead to novel approaches for targeting Stat3 with specific inhibitors, and the development of new strategies for therapeutic intervention in breast cancer metastasis.

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